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# Repositionnement de molécules médicamenteuses dans la lutte contre les bactéries multi-résistantes

Lucie Peyclit

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Par Mme **Lucie PEYCLIT**  
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**TITRE :**

**Repositionnement de molécules médicamenteuses  
dans la lutte contre les bactéries multi-résistantes**

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*« L'Université n'entend donner aucune approbation, ni improbation aux opinions émises dans les thèses. Ces opinions doivent être considérées comme propres à leurs auteurs. »*

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## II. Introduction

La résistance bactérienne aux antibiotiques est un problème majeur de santé publique selon l’OMS qui a adopté un plan d’action mondial pour sa lutte en 2015<sup>1</sup>. Bien que ce phénomène ne soit pas nouveau, nous avons désormais les moyens techniques de constater qu’elle a évolué partout dans le monde et dans plusieurs écosystèmes via l’influence de l’utilisation d’antibiotiques chez les animaux (lors de la production agricole) et chez l’homme (avec les infections associées aux soins de santé)<sup>2,3</sup>. Il est difficile d’en estimer le réel impact dû à l’utilisation de multiples définitions et au manque de données empiriques<sup>4</sup>. Les estimations actuelles de la résistance bactérienne ne sont pas très fiables et requièrent des données plus détaillées<sup>5,6</sup>. La compréhension de ces mécanismes, de ces facteurs de risque et de l’épidémiologie reste primordiale pour la réussite de la lutte contre les infections aux bactéries résistantes et pour ainsi orienter les cliniciens vers des thérapies plus adaptées<sup>7</sup>.

Ces dernières années, la prévalence de la résistance bactérienne semble être davantage rapportée chez les bactéries à Gram négatif (BGN) telles que les entérobactéries ou les bacilles non fermentants (*Pseudomonas aeruginosa*, *Acinetobacter baumannii*)<sup>8,9</sup>. Dans certains cas, ces agents pathogènes ont exprimé une résistance à plusieurs molécules cliniquement disponibles, se qualifiant ainsi de multirésistants<sup>10</sup>. C’est donc avec l’émergence d’organismes producteurs de  $\beta$ -lactamases à spectre étendu (BLSE) que les cliniciens ont eu recours dans un premier temps aux carbapénèmes, des antibiotiques à large spectre et résistants à l’hydrolyse de ces enzymes. Malheureusement, ceci a conduit depuis plusieurs années à une augmentation de la résistance à ces molécules clés<sup>11</sup>. Cette résistance peut résulter de divers mécanismes comme la modification de la perméabilité membranaire ou la production par la bactérie de carbapénémases telles que KPC, NDM ou des oxacillinases (OXA-48, OXA-23, OXA-24)<sup>12</sup>.

En parallèle, l’Age d’or de l’antibiotique semble sur le déclin en raison de la diminution du développement de nouveaux antibiotiques<sup>13</sup>. Des progrès majeurs ont largement contribué à la diminution des décès dus aux maladies infectieuses. Les antibiotiques ne faisant pas partie des stratégies de traitement des maladies chroniques, le développement de nouvelles solutions thérapeutiques devient donc moins intéressant économiquement pour l’industrie pharmaceutique<sup>14</sup>. Du côté clinique, la tendance a donc été de réutiliser des molécules plus anciennes et encore disponibles comme traitement de dernier recours. Ces molécules, comme les polymyxines qui furent abandonnées à cause de leur toxicité rénale et neurologique notamment, s’avèrent efficaces contre des bactéries résistantes aux carbapénèmes<sup>15</sup>.

La polymyxine B et la polymyxine E, ou colistine, sont des peptides antimicrobiens, synthétisés par *Paenibacillus polymyxa* découverts dans les années 1950<sup>16</sup>. Elles agissent au niveau de la membrane

cellulaire externe des BGN. La colistine se lie aux lipopolysaccharides (LPS) et déplace de manière compétitive les cations de ces lipides membranaires, ce qui entraîne une perturbation de la membrane cellulaire. Il s'en suit une fuite du contenu intracellulaire et donc la mort bactérienne.

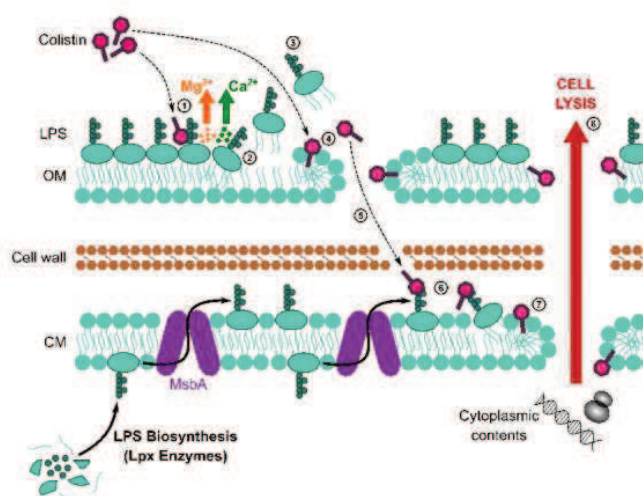


Figure 1. Mécanisme d'action de la colistine<sup>17</sup>

La colistine est active contre de nombreuses bactéries multi-résistantes (BMR) à Gram négatif et est utilisée pour traiter les infections pulmonaires sévères à *Klebsiella pneumoniae*, *A. baumannii* ou *P. aeruginosa* chez les patients atteints de mucoviscidose par exemple<sup>18</sup>. De plus, elle est largement utilisée en médecine vétérinaire dans l'élevage animal<sup>19</sup>. Le taux de résistance à cet antibiotique est en augmentation à travers le monde et plusieurs mécanismes complexes conférant une résistance de haut ou faible niveau ont été décrits. Par la synthèse de sucres, ils entraînent principalement une modification du LPS diminuant l'affinité de la colistine, qui devient inefficace. Ils peuvent être d'origine chromosomique ou plasmidique<sup>20</sup>. Chez les entérobactéries, les mécanismes de résistance fréquemment rencontrés correspondent à une inactivation du gène *mgrB* ou des modifications de gènes régulateurs (*pmrAB*, *phoPQ*). Les concentrations minimales inhibitrices (CMI) à la colistine de ces souches sont généralement comprises entre 4 et 64 mg/l<sup>21</sup>. L'émergence d'une résistance plasmidique à la colistine et transférable d'une espèce à une autre a été rapportée pour la première fois fin 2015 avec le gène *mcr-1*, neuf autres variants ayant été décrits depuis le gène *mcr-10* en 2020<sup>22</sup>. La protéine MCR confère un bas niveau de résistance avec des CMI à la colistine plus faible<sup>20</sup>. De nombreuses études ont rapporté la dissémination de ces souches résistantes sur tous les continents chez les animaux d'élevage, dans des produits alimentaires mais aussi plus rarement chez l'homme<sup>23</sup>.

L'émergence et la dissémination de la résistance à la colistine sont préoccupantes car cette molécule fait partie de l'une des dernières lignes thérapeutiques efficaces sur les BMR. Les cliniciens se retrouvent contraints de traiter des infections à des germes multi-résistants avec un arsenal antimicrobien limité. En effet, Monaco et co-auteurs. ont montré en Italie que sur 191 souches cliniques, 178 (93%) des

*K. pneumoniae* présentaient des enzymes KPC (carbapénémases), dont 76 (43%) étaient respectivement résistantes à la colistine<sup>24</sup>. Par définition, les BMR sont résistantes à  $\geq 1$  agent dans  $\geq 3$  catégories d'antimicrobiens, les bactéries avec une résistance étendue aux médicaments (XDR) restent sensibles à une ou deux catégories seulement et les pan-résistantes (PDR) sont résistantes à tous les agents antimicrobiens testés en routine<sup>10</sup>. Ceci permet alors d'évaluer l'émergence de ces souches et le potentiel thérapeutique encore disponible pour les traiter. Toutefois, il semblerait que ces définitions surestiment le nombre de BMR car elles bannissent une classe thérapeutique à partir du moment où la bactérie est résistante à un agent de cette classe. Pourtant, les cliniciens utilisent souvent d'autres antibiotiques actifs dans une même classe, surtout lorsque les options sont peu nombreuses. C'est ainsi qu'un autre système de classification de la résistance aux BGN a vu le jour, appelé « résistance difficile à traiter » ou *difficult-to-treat* en anglais (DTR)<sup>25</sup>. Elle repose sur le fait de savoir si des antibiotiques de première ligne et faiblement toxiques sont encore efficaces pour traiter l'infection. Elle est donc définie comme la résistance in vitro à toutes les  $\beta$ -lactamines, y compris les carbapénèmes, et aux fluoroquinolones<sup>7</sup>. En 2018, la revue de la littérature de Abat C. et co-auteurs. a identifié 14 décès humains dus aux bactéries PDR à partir d'une large recherche documentaire et seulement un seul parmi les patients ayant eu des infections dans notre hôpital à Marseille. Ceci est très divergent des prévisions alarmistes<sup>5</sup> et montre la discordance existant entre les données factuelles et celles extrapolées. Les impasses thérapeutiques lors d'infections à des bactéries résistantes restent peu fréquentes à ce jour mais il est vrai que dans certaines situations, les cliniciens font face à des infections difficiles à traiter chez des patients fragiles, ayant reçus de longs traitements antibiotiques<sup>26</sup>. En outre, les industries pharmaceutiques y portent peu d'intérêt et les seuls candidats antibiotiques actuellement en cours de développement présentent au moins une certaine résistance croisée avec les agents existants<sup>27</sup>. Ainsi, des alternatives thérapeutiques aux antimicrobiens classiques doivent être étudiées pour trouver de nouvelles armes contre ces pathogènes résistants.

De nombreuses pistes sont donc envisagées pour lutter contre ces infections allant de la greffe de microbiote fécal aux bactériophages, des virus infectant les bactéries, jusqu'à l'immunisation passive avec l'injection d'anticorps contre les facteurs de virulence bactériens<sup>28</sup>. Une stratégie a notamment retenu notre attention et a fait l'objet de ces travaux : le repositionnement moléculaire. Ce concept consiste à utiliser un médicament déjà approuvé dans une nouvelle indication. En d'autres termes, le but est de cibler une autre pathologie ou cible pour laquelle cette molécule est déjà utilisée tout en ayant connaissance des données pharmacocinétiques et toxicologiques<sup>29</sup>. Le sujet de cette thèse est donc le suivant : le repositionnement de molécules médicamenteuses dans la lutte contre les BMR. L'objectif est d'étudier cette stratégie innovante avec une mise en pratique simple pour une application dans le traitement des infections à des BMR.

Nous avons initié ce projet par une revue de la littérature scientifique permettant d'obtenir une bibliographie exhaustive sur ce sujet. Nous y avons défini le terme et les méthodes du repositionnement moléculaire puis nous avons établi un état des lieux des molécules repositionnées efficaces contre les bactéries résistantes aux carbapénèmes et à la colistine. Nous avons abordé leur efficacité dans le cadre des combinaisons médicamenteuses et leur utilisation potentielle en clinique (**Article 1**).

Dans un second temps, dans le cadre de recherche d'une stratégie alternative, nous avons réalisé un criblage *in vitro* d'une bibliothèque de 1280 molécules approuvées par la *Food and Drug Administration* (FDA) sur une collection de BMR cliniques. L'objectif était d'étudier l'inhibition de la croissance de différentes bactéries issues d'un panel présentant des mécanismes de résistances variés. Nous avons ainsi rassemblé deux bactéries de type cocci à Gram positif (GP) résistants aux glycopeptides (*Enterococcus faecium*, *Staphylococcus aureus*), six à Gram négatif dont deux entérobactéries résistantes aux carbapénèmes et à la colistine (*K. pneumoniae*, *Escherichia coli*) et quatre bacilles non fermentants qualifiées de XDR (*A. baumannii*, *P. aeruginosa*, *Burkholderia multivorans*, *Pandoraea nosoerga*). Nous avons sélectionné les composés avec la meilleure efficacité pour chaque bactérie et étudié leur application possible en clinique au vue des données de la littérature (**Article 2**). A l'hôpital, les cliniciens font parfois face à de graves infections bactériennes chez des personnes immunodéprimées tels que les patients greffés pulmonaires atteints de mucoviscidose. Ces patients ont souvent reçu des protocoles d'antibiothérapies répétés et de longue durée, et leurs germes présentent des résistances acquises nombreuses, constituant un défi pour leur traitement. Dans ce cadre-là, l'utilisation de notre criblage *in vitro* au laboratoire peut être mis en pratique, offrant alors une réponse personnalisée, rapide et efficace pour le clinicien. Nous avons étudié une souche de *Pandoraea nosoerga*, issue d'une patiente atteinte de mucoviscidose et hospitalisée dans notre institut, par une approche génomique après l'avoir testée phénotypiquement à notre banque de molécules (**Article 3**).

Enfin, nous nous sommes concentrés sur un hit prometteur trouvé lors du criblage, la zidovudine (AZT). Des données pré-cliniques ainsi que nos travaux préliminaires de screening ont permis d'évaluer l'efficacité de l'AZT, initialement classée et utilisée comme un antirétroviral, contre les bactéries résistantes aux carbapénèmes et à la colistine. Un panel varié d'entérobactéries résistantes à ces molécules de dernière ligne a montré des CMI d'AZT entre 0,06 mg/l et 1,7 mg/l. Ces concentrations sont compatibles avec les taux sériques retrouvées lors d'une utilisation en clinique et montrent un véritable intérêt de cette molécule pour le traitement des infections à ces BMR (**Article 4**). Parallèlement à ceci, nous avons isolé des souches cliniques provenant de notre hôpital avec une CMI élevée à l'AZT ce qui nous a permis d'établir un niveau de prévalence de la résistance à l'AZT (défini à 26,7 mg/l d'après les données cliniques et la répartition des CMI de l'AZT chez ces espèces) à 6.7%. Le travail consistait également à caractériser et comprendre le support moléculaire de la résistance. La thymidine

kinase, une enzyme supposée activer l'AZT dans la cellule, fut notre première cible via l'étude des séquences de souches résistantes. Puis, une analyse génomique comparative de 633 génomes complets d'*E. coli* a été réalisée dans le but d'étudier la diversité de la thymidine kinase. L'apparition rapide de la résistance après exposition des souches à l'AZT suggère son utilisation en combinaison plutôt qu'en monothérapie, et confirme la nécessité de recourir à des tests de sensibilité au laboratoire de diagnostic (**Article 5**).

Puis, dans le cadre d'un stage hors-subdivision (inter-CHU) d'un an et donc une collaboration avec Monash University (Melbourne, Australie) dans l'équipe du Pr. Jian Li, nous avons évalué l'efficacité de la combinaison colistine et AZT contre des souches de *K. pneumoniae* sensible ou résistante à la colistine. Leurs connaissances et leurs moyens en termes de pharmacocinétique et de pharmacodynamie (PK/PD) nous ont permis d'approfondir nos recherches pour cette molécule. En effet, l'association offre une thérapeutique prometteuse pour le traitement des entérobactéries multi-résistantes (**Article 6**).



### III. Repositionnement moléculaire en vue du traitement des bactéries résistantes à la colistine et aux carbapénèmes

Le développement d'antibiotiques peut être long, fastidieux et coûteux. Face à de nouvelles menaces, des stratégies alternatives sont envisagées et les esprits scientifiques se dirigent vers des thérapeutiques innovantes<sup>30</sup>. Une option prometteuse est le repositionnement des médicaments ou *drug repurposing* en anglais. Celui-ci consiste à utiliser des molécules approuvées, déjà connues pour une indication, afin de traiter de manière inattendue une autre pathologie. Les principaux avantages sont le renouvellement de médicaments anciens et oubliés, une réduction des coûts, une rapidité de développement et la connaissance au préalable des propriétés pharmacologiques et toxiques. Divers travaux ont été réalisés ces dernières années à ce sujet aussi bien en cancérologie<sup>31</sup>, en neurologie<sup>32</sup> ou en infectiologie<sup>33</sup>. Cette reconversion thérapeutique est essentielle pour offrir de nouvelles solutions aux infections par des micro-organismes résistants par exemple<sup>34</sup> mais aussi pour apporter une réponse rapide à une épidémie ou à l'émergence de nouveaux pathogènes comme tout récemment avec le nouveau coronavirus SARS-CoV-2<sup>35,36</sup>.

Dans cette revue, nous nous sommes focalisés sur le repositionnement des médicaments contre des bactéries fréquemment rencontrées en pratique clinique et résistantes à des antibiotiques utilisés en dernière ligne<sup>8,37</sup> : les BGN résistantes à la colistine et aux carbapénèmes. L'objectif de cette revue était d'identifier les précédents travaux réalisés et les méthodes engagées puis de recenser les principales molécules ayant été identifiées pour lutter contre les infections à ces bactéries résistantes.

Dans un premier temps, nous avons présenté les principales définitions, ainsi que les avantages et les inconvénients de cette stratégie. Trois méthodes ont été abordées pour la mettre en œuvre : phénotypique, informatique et par « hasard ».

Dans un deuxième temps, grâce à une étude de la littérature, les diverses molécules déjà identifiées ont été rassemblées en fonction de leur classe thérapeutique d'origine et leur activité bactérienne. Le ciclopirox, le gallium, la pentamidine et la zidovudine ont notamment attiré notre attention. Nous avons également montré que le repositionnement pouvait être inclus dans des combinaisons thérapeutiques, ce qui améliorerait l'efficacité antibactérienne d'une molécule par effet synergique.

Enfin, nous avons étudié les perspectives de cette stratégie face au désintérêt des industries pharmaceutiques mais aussi avec le regard que pourrait avoir les cliniciens quant à l'utilisation de molécules dans une autre indication que celle préconisée.

**1. Article 1. *Drug repurposing to fight colistin and carbapenem-resistant bacteria* - Peyclit L., Baron SA., et Rolain J-M.**

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# Drug Repurposing to Fight Colistin and Carbapenem-Resistant Bacteria

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The emergence of new resistance mechanisms, the failure of classical antibiotics in clinic, the decrease in the development of antibiotics in the industry are all challenges that lead us to consider new strategies for the treatment of infectious diseases. Indeed, in recent years controversy has intensified over strains resistant to carbapenem and/or colistin. Various therapeutic solutions are used to overcome administration of last line antibiotics. In this context, drug repurposing, which consists of using a non-antibiotic compound to treat multi-drug resistant bacteria (MDR), is encouraged. In this review, we first report what may have led to drug repurposing. Main definitions, advantages and drawbacks are summarized. Three major methods are described: phenotypic, computational and serendipity. In a second time we will focus on the current knowledge in drug repurposing for carbapenem and colistin-resistant bacteria with different studies describing repurposed compounds tested on Gram-negative bacteria. Furthermore, we show that drug combination therapies can increase successful by drug repurposing strategy. In conclusion, we discuss the pharmaceutical industries that have little interest in reprofiling drugs due to lack of profits. We also consider what a clinician might think of the indications of these uncommon biologists to treat MDR bacterial infections and avoid therapeutic impasses.

**Keywords:** repurposing, multi-drug resistance (MDR), bacteria, colistin resistance, carbapenem resistant enterobacteriaceae

## INTRODUCTION

Nowadays, despite recent scientific progress, infectious diseases must always be taken into consideration. The World Health Organization (WHO) closely examines such concerns in order to have an effective health system (World Health Organization, 2018). For 50 years, we have been confronted with the end of the golden age of antibiotic discovery, while some antimicrobial substances have existed for years (Gould, 2016). Due to significant progress that has largely contributed to reducing the number of deaths from infectious diseases, pharmaceutical companies have developed a decreasing interest in these drugs (Conly and Johnston, 2005).

In addition, the use of an antibiotic and the emergence of its resistance are inevitable and intrinsically linked (Mohr, 2016). Although this is not a new phenomenon but a natural one, WHO analysis warns against this serious situation which is the impact, nature and spread of global antimicrobial resistance (Global Antimicrobial Resistance Surveillance System, 2019 Report Early implementation). These resistant bacteria are found in every kind of environment: water, animals, humans, plants and food (Rolain, 2013; Zenati et al., 2016; Bachiri et al., 2017; Tafoukt et al., 2017). The inappropriate use of antimicrobial agents and the spread of antibacterial resistance are among factors that lead to a high rate of resistance in clinical, animals, and even in environmental isolates

(Roca et al., 2015; Bassetti et al., 2017). Partly because of drug pressure, resistance can occur more easily and affect all types of antibiotics as for the last-line antibiotics used in human medicine drug for resistant bacterial infections (Biswas et al., 2012). In recent years, we have seen an increase in the use of carbapenems as a result of an increase in the carbapenem resistance of Gram-negative bacteria (GNB) (Diene and Rolain, 2013). For example, Monaco et al. showed in Italy that among 191 clinical strains isolated from November 2013 to April 2014, 178 (93%) *Klebsiella pneumoniae* had KPC enzymes (carbapenemases), with 76 (43%) resistant to colistin (Monaco et al., 2014). Although the same situation has been reported with colistin (Olaitan et al., 2014), it has received more attention: last-line treatments may no longer be effective, increasing the risk of spreading infections (Biswas et al., 2012). To combat frequent epidemics and the challenge of rapid spread, new alternatives to last resort treatments must be considered to avoid treatment failure.

As a result, alternatives to antibiotics to treat resistant germs should be a priority (Bassetti et al., 2017). The use of old drugs can be a solution like “forgotten” antibiotics polymyxins, fosfomicin, minocycline or mecillinam, which are still used in clinical settings (Cassir et al., 2014). There is also a renewed interest in antibiotic combinations to circumvent resistance (Lenhard et al., 2016). For example, the synergistic activity of sulfonamide-associated colistin was evaluated against colistin-resistant clinical bacteria (Okdah et al., 2018). But “non antibiotic” solutions can also be considered as alternatives for the therapeutic management of infections (Aslam et al., 2018). Various studies showed that *Clostridium difficile* can be inhibited using bacteriophages or several ongoing trials use antimicrobial peptides as alternatives or preventive treatments in the future (Aslam et al., 2018).

The fight to treat multi-drug resistant (MDR) infections must also include a change in mentality. Rolain and Baquero denounced the fact that society does not accept the use of toxic but effective antibiotics in treatment of life-threatening infections, but on the other hand society can tolerate potential toxicities of other drugs, such as anti-cancer. With the progress of medicine in the management of adverse reactions and the improved monitoring of antibiotic concentrations, old drugs or dosages rejected due to their adverse effects have to be reconsidered (Rolain and Baquero, 2016). In this way, one other promising alternative on which this review focuses is drug repurposing, also called repositioning (Mercorelli et al., 2018). This therapeutic shift is the subject of several studies in different pathologies including cancer (Sleire et al., 2017), heart diseases (Sun et al., 2018), Alzheimer’s disease (Kim, 2015) or depression (Ebada, 2017).

In infectiology, repurposing studies are now being carried out (Torres et al., 2016; Soo et al., 2017; D’Angelo et al., 2018; Zheng et al., 2018; Miró-Canturri et al., 2019). In general, the most common bacteria are first tested or those most at risk or in a therapeutic deadlock. If this review focuses on drug repurposing that have been tested on MDR bacteria, it seems important to precise that resistance is rarely crossed and if a molecule is active on a specific species, this compound will potentially be active regardless of its resistance mechanisms. This is because this molecule affects a new target, generally

independent of the antibiotic target, as we will see below with ciclopirox (Carlson-Banning et al., 2013), gallium (Goss et al., 2018), and zidovudine (Elwell et al., 1987). Therefore, it can expand the scope to combinations tested on sensitive GNB as for minocycline and polymyxin B tested with non-antibiotics drugs (Schneider et al., 2017). It offers a diversified and still exploitable field of possibilities (Schneider et al., 2017). For carbapenem and colistin-resistant isolates, a few articles are published on this specificity for which we are striving to synthesize them. The aim is thus to identify an innovative therapeutic strategy against these bacteria in a cost-effective and efficient way.

In this review, we will define drug repurposing and its characteristics. We will then make an inventory of what has already been published as a drug for reuse in general and in particular to address the problem of carbapenem and colistin-resistant bacteria. Finally, we will see what prospects exist for this therapeutic strategy.

## DISCOVERY OF AN ANTIBACTERIAL POTENTIAL IN NON-ANTIBIOTIC DRUGS

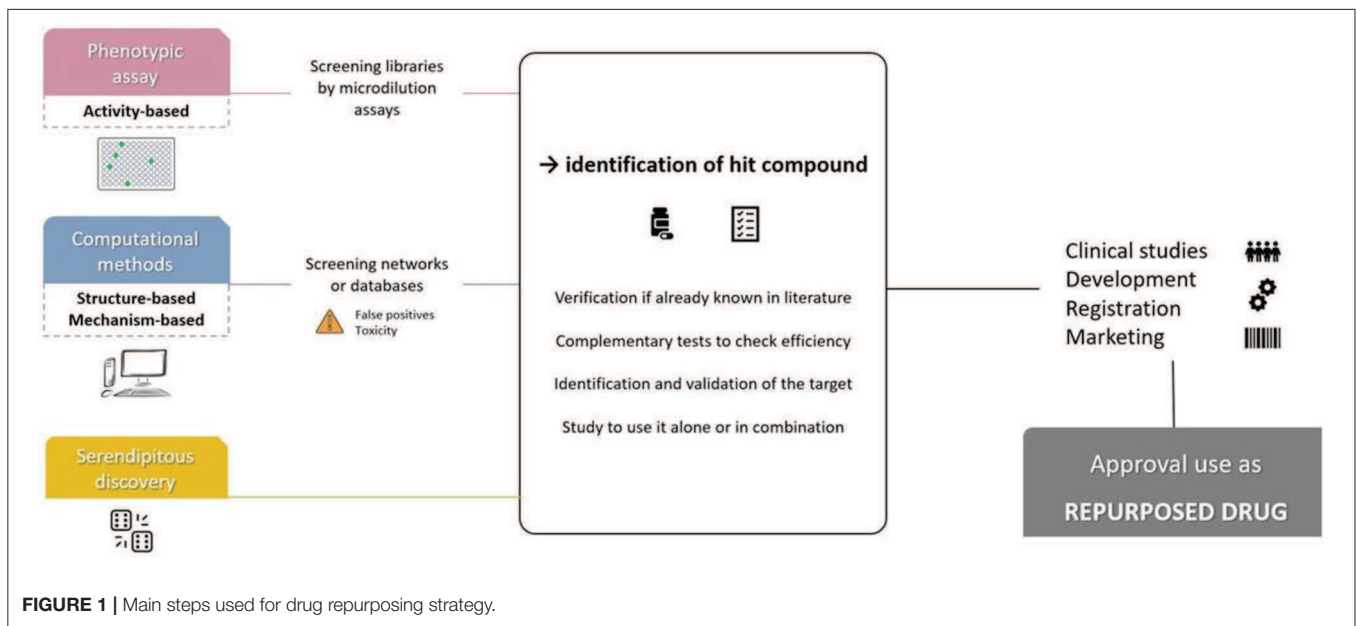
### What Does Drug Repurposing Mean?

A drug class is assigned to a molecule to describe and group similar together drugs because of their therapeutic use, their biochemical mechanism, by their way of action or their chemical structure. As defined by Waksman in 1947, “an antibiotic is a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms” (Mohr, 2016). The current trend therefore seems to be moving away from this definition. Indeed, in recent years, drug repositioning seems to have been “a promising field in drug discovery that identifies new therapeutic opportunities for existing drugs” (Doan et al., 2011). The common idea is that to accelerate discovery of new treatment, using old drugs that could potentially treat disease for which the treatments used no longer work or when we no longer have therapeutic solutions must be used (Langedijk et al., 2015). Sir James Black, pharmacologist and Nobel laureate said in 1888: “The most fruitful basis for the discovery of a new drug is to start with an old drug” (Chen et al., 2016). It could help to overcome an initial bottleneck in drug development process. It may therefore be a better compromise between risk and reward than other approaches to drug development (Ashburn and Thor, 2004).

### What Are Advantages and Drawbacks?

Drug repurposing present real economic advantages. All studies about structure, pharmacological properties as bioavailability or safety profiles for example have already been conducted. With these drugs, it is possible to skip preclinical trials because toxicity and pharmacokinetic are already known and a certain hindsight has been taken for several years. Drugs can move directly to Phase 2 to test their effectiveness (Mercorelli et al., 2018).

Repurposing drugs can offer new pathways or targets to study new perspectives for curing diseases. As many antibiotics already affect DNA, membrane or protein translation, other pathways essential for bacterial growth, remain available for activity of molecules, such as assimilation pathways of essential compounds



like sugars or amino acids. Combined strategies that reduce resistance can be used to achieve several targets that could affect bacterial metabolism (Mercorelli et al., 2018; Zheng et al., 2018).

Moreover, this method is favorable to academic or small laboratories because of disinterest of pharmaceutical industries. Without patents, these industries do not see any fruitful interest in it because of rapid emerging resistance (Fernandes and Martens, 2017) and a narrower spectrum of activity (Zheng et al., 2018).

On the other hand, this solution cannot be totally miraculous. Drug repurposing does not work all the time due to the high minimal inhibitory concentration (MIC) (Mercorelli et al., 2018) or inconsistent plasma concentrations tolerated in humans. Dose tested for this new indication is important and can lead to human toxicity (Zheng et al., 2018), what society fears (Rolain and Baquero, 2016). Concerning galenic, an optimization of formulation can also be foreseen if a physico-chemical incompatibility is observed.

## How to Process to Repurpose Drugs?

Considered an innovative strategy (Doan et al., 2011), three major methods can lead a drug to be repurposed, as shown in Figure 1.

First, phenotypic assay can be performed by high throughput and screening of commercial, public, pharmaceutical compound libraries (Jung et al., 1997; Kim, 2015). These assays consist in classical broth microdilution to identify a hit (Torres et al., 2016). Once antibacterial activity is found, MIC assay is performed to confirm results according to CLSI guidelines (Sun et al., 2016b). Compared to other methods, phenotypic tests have the advantage of being highly physiologically relevant because the effect is observed directly on bacteria (Zheng et al., 2018).

Advances in genomics and bioinformatics modified drug repositioning approach. It consists of *in silico* structure or mechanism-based assays that work with virtual databases. This

has been made possible through the prospective development of drug databases and activities, the exchange of information on compounds in collaborative networks and the abundance of resources on the Internet (Hodos et al., 2016). These new calculation tools make it possible to analyse all the different data accumulated in the field that man alone cannot study because they are too complex. This can ensure the understanding and prediction of molecules by generating hypotheses about biological mechanisms (Hodos et al., 2016). Limit to these approaches is that pathways, targets or other data must be already known. Moreover, false positive and toxicity are problematic issues found after identification of a hit (Mercorelli et al., 2018). However, for emerging diseases, this could be a quick way to find an effective molecule as was done for the coronavirus in 2013 (Law et al., 2013).

The last approach is random discovery and can include all types of configurations. Indeed, the side effects of one drug in one disease may be effective for another, such as for the antidepressant bupropion reoriented as an anti-tobacco drug (Hodos et al., 2016). It can be mere coincidence as sulfamides were known for antibacterial properties and finally also employed for antidiabetic ones (Deuil, 1956). However, these unexpected observations could also potentially be identified by informatics methods, in view of knowledge of compound side effects (Hodos et al., 2016).

Despite all these techniques, if a molecule is identified, it must then go through the steps of its clinical evaluation.

## CURRENT KNOWLEDGE IN DRUG REPURPOSING FOR CARBAPENEM AND COLISTIN RESISTANCE?

Studies generally screen MDR bacteria to ensure a broader spectrum of action (Hijazi et al., 2018) and sometimes bacteria

**TABLE 1** | Relevant repurposing reports for carbapenem and colistin resistant bacteria.

	<b>Compound</b>	<b>Approved use or known as</b>	<b>Activity -alone or in combination with-</b>	<b>Tested bacteria</b>	<b>Resistance phenotypes (and/or)</b>	<b>References</b>
Infectiology	Zidovudine	Antiretroviral	Alone	<i>Escherichia coli</i> <i>Klebsiella pneumoniae</i>	Carbapenems Colistin	Peyclit et al., 2018
			Colistin	<i>E. coli</i> <i>K. pneumoniae</i> <i>Enterobacter cloacae</i> <i>Pseudomonas aeruginosa</i> <i>Acinetobacter baumannii</i>	Carbapenems Colistin	Hu et al., 2018; Loose et al., 2018
			Tigecycline	<i>E. coli</i> <i>K. pneumoniae</i>	Carbapenems	Ng et al., 2018
	Nicosamide	Anthelmintic	Colistin	<i>A. baumannii</i> <i>K. pneumoniae</i>	Colistin	Ayerbe-Algaba et al., 2018
	Pentamidine	Antiprotozoal	Alone			Cabrero-Cangueiro et al., 2018
Rifampicin			<i>K. pneumoniae</i> <i>E. coli</i> <i>E. cloacae</i>	Carbapenems Colistin	Cabrero-Cangueiro et al., 2018	
Ciclopirox	Antifungal	Aminoglycosides			Cabrero-Cangueiro et al., 2018	
		Alone	<i>E. coli</i> <i>K. pneumoniae</i> <i>A. baumannii</i>	Carbapenems	Carlson-Banning et al., 2013	
Oncology	5-fluorouracil	Antineoplastic	Zidovudine	<i>A. baumannii</i>	Carbapenems	Cheng et al., 2019
	Mitotane	Antineoplastic	Polymyxin B	<i>A. baumannii</i> <i>P. aeruginosa</i> <i>K. pneumoniae</i>	Carbapenems Polymyxin	Tran et al., 2018
	Gallium	Antineoplastic	Alone	ESKAPE species	MDR	Hijazi et al., 2018
	Tamoxifen Raloxifen Toremifen	SERM	Polymyxin B	<i>P. aeruginosa</i> <i>K. pneumoniae</i> <i>A. baumannii</i>	Colistin	Hussein et al., 2017
Central Nervous System	Sertraline	Antidepressant	Polymyxin B	<i>P. aeruginosa</i> <i>K. pneumoniae</i>	Colistin Carbapenems	Otto et al., 2019
	Citalopram	Antidepressant	Polymyxin B	<i>A. baumannii</i> <i>E. coli</i> <i>K. pneumoniae</i>	Colistin Carbapenems	Otto et al., 2019
	Fluspirilene	Antipsychotic	Colistin	<i>A. baumannii</i>	Carbapenems	Cheng et al., 2019
Metabolism	Bay 11-7082	Anti-inflammatory	Colistin	<i>A. baumannii</i>	Carbapenems	Cheng et al., 2019
	Spirolactone	Diuretic	Polymyxin B	<i>E. coli</i>	Carbapenems	Otto et al., 2019
Natural compound	Resveratrol	Stilbene	Alone	<i>E. coli</i> <i>Enterobacter aerogenes</i>		Seukep et al., 2016
			Streptomycin	<i>K. pneumoniae</i>	MDR	Seukep et al., 2016
			Ciprofloxacin	<i>P. aeruginosa</i> <i>Providencia stuartii</i> <i>E. cloacae</i>		Seukep et al., 2016
			Colistin	<i>E. coli</i> <i>K. pneumoniae</i> <i>A. baumannii</i> <i>Serratia marcescens</i> <i>Proteus mirabilis</i>	Colistin Carbapenems	Cannatelli et al., 2018
	Pterostilbene	Anticancer Antioxidant	Polymyxin B	<i>K. pneumoniae</i>	Colistin	Zhou et al., 2018
	Eugenol	Essential oil	Colistin	<i>E. coli</i>	Colistin	Wang et al., 2018

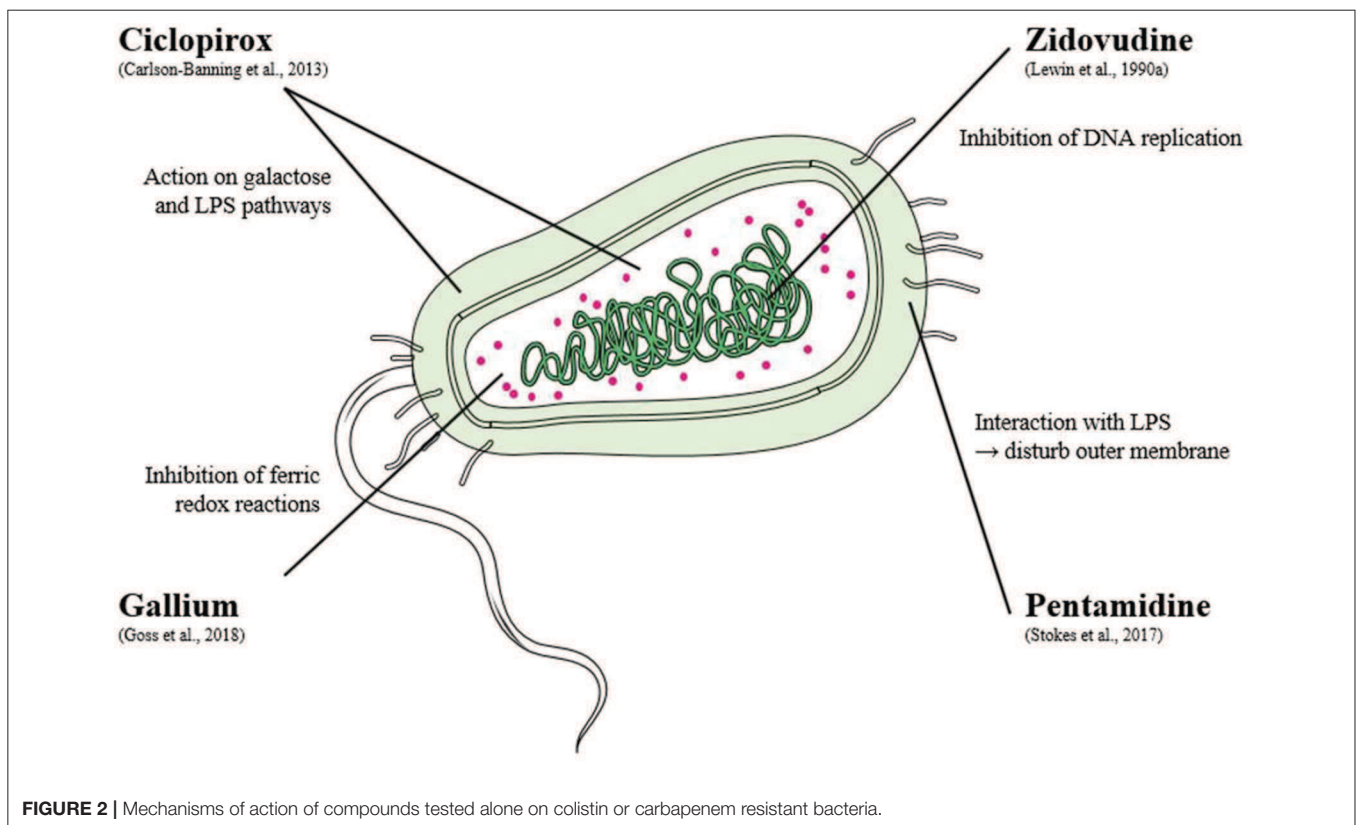
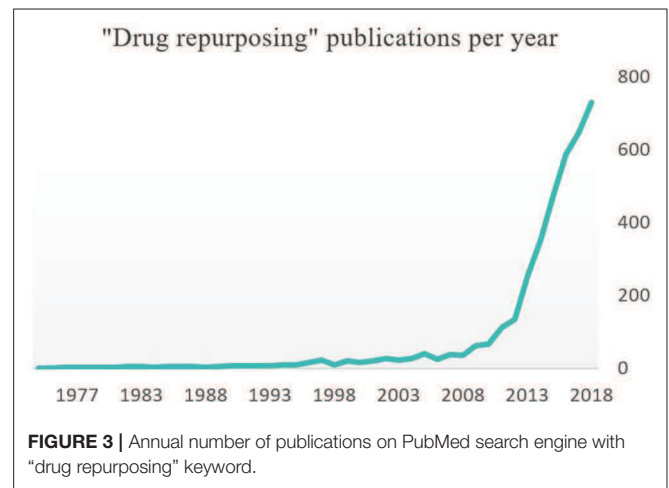
SERM, Selective estrogen receptor modulator.



only with colistin or carbapenem resistance to treat the ongoing issue (Ayerbe-Algaba et al., 2018). Several major studies have performed drug repurposing on MDR bacteria belonging to the ESKAPE (*Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species) group (Table 1). Seven non-antibacterial compounds inhibited the growth of an *Acinetobacter baumannii* strain resistant to most antibiotics including carbapenems: 3 antineoplastics (5-fluorouracil, 6-thioguanine and pifithrin- $\mu$ ), 1 anti-rheumatic (auranofin), 1 antipsychotic (fluspirilene), 1 anti-inflammatory (Bay 11-7082), and 1 alcohol deterrent (disulfiram). Five-fluorouracil and 6-thioguanine seemed to be the best candidates for repurposing to treat MDR clinical *A. baumannii*. Their IC90 values or MIC were lower than standard plasma drug concentration levels in human, suggesting a possible use without major adverse events (Cheng et al., 2019).

All mechanisms of action and targets are considered because the objective is to escape therapeutic drug classes. Each repurposed molecule can be used to study a new pathway (Figure 2). An antifungal agent developed nearly forty years ago, ciclopirox, also has good repurposing criteria, as shown by an American study conducted in 2013 (Carlson-Banning et al., 2013). Due to its excellent safety profile, it has already been repurposed in various pathologies such as myeloma, or as an anti-human immunodeficiency virus drug. It prevents enzyme actions, essential for cellular metabolism or functions, by inhibiting the availability of co-factors. Its activity was proved against MDR *E. coli*, *K. pneumoniae*, and *A. baumannii* strains.

They demonstrated a novel mechanism of action: ciclopirox affects the galactose and LPS salvage pathways (Carlson-Banning et al., 2013). In addition, the bacterial activity of gallium has been known for more than 80 years but is first used as an anti-cancer agent. Due to its chemical similarity to iron, gallium inhibits ferric redox reactions or pathways, and then bacterial growth. In this matter, it has a broad spectrum of activity, in particular MDR ESKAPE pathogens (Rangel-Vega et al., 2015; Hijazi et al., 2018). In fact, a phase 2 trial in cystic fibrosis patients assess the activity of gallium and suggests its safety and



efficacy for human infections (Goss et al., 2018). This once again represents potential and promising targets for the control of infectious germs.

It can be noticed that all pharmaceutical classes can be involved, from anticancer to anti-inflammatory and also antiparasitic drugs. Pachón-Ibáñez et al. and the study of Stokes (Stokes et al., 2017) of the previous year showed that pentamidine was effective against polymyxin resistant strains. This antiprotozoal agent usually effective in trypanosomiasis, leishmaniasis and some fungal infections was here tested against 8 *Enterobacteriaceae* (5 *K. pneumoniae*, 1 *E. coli* and 2 *Enterobacter cloacae*). Pentamidine was bactericidal for 7 strains which carried out carbapenemases or showed colistin resistance. Moreover, these effects potentiated activity of other antibiotics due to a synergistic activity with rifampicin, or aminoglycosides for *E. cloacae*. With rifampicin, combination was effective against most of the strains tested (Cebrero-Canguero et al., 2018).

In this context of drug reprofiling, various studies identified the antiretroviral zidovudine, also called azidothymidine (AZT), as an active molecule against resistant *Enterobacteriaceae* (Doléans-Jordheim et al., 2011; Peyclit et al., 2018). The interest in drug repurposing in MDR enterobacterial infections has revived the forgotten antibacterial properties of this drug mentioned for the first time in 1986 (Elwell et al., 1987). On a series of *Enterobacteriaceae* with different colistin resistance profiles (*mcr-1* gene, *mgrB* or *pmrB* mutations), its antibacterial action was confirmed with MICs ranging from 0.2 and 6.25  $\mu$ M. Pharmacokinetic data showed that AZT concentrations found would be compatible with plasma concentrations obtained for doses used in human medicine (Peyclit et al., 2018). Due to a relatively rapid mutation frequency (Doléans-Jordheim et al., 2011) and resistant strains already reported (Lewin et al., 1990a), it would appear that zidovudine is more suitable for use in combination. Indeed, zidovudine was tested in various associations with antibiotics from different class (Lewin et al., 1990b; Mascellino et al., 1993). In a recent article (Hu et al., 2018), checkerboard analysis with colistin showed synergistic activity against 60.87% of the Extended spectrum  $\beta$ -lactamases (ESBL) *E. coli*, 87.1% of the ESBL *K. pneumoniae*, 100% of NDM-1 producing strains and 92.31% of colistin resistant (*mcr-1*) *E. coli*. With this bactericidal combination, the activity of colistin has been improved, which could reduce the dose of colistin for a better effect (Hu et al., 2018). Patented in 2014 (Hu and Coates, 2014), it has been enrolled in a Phase 1 clinical trial. Results showed that the association had a bactericidal activity on plasma concentration on *mcr-1* positive strains and that it was well tolerated by the healthy volunteers involved in the study (Loose et al., 2018). Further human studies can be undertaken to confirm these results, but they confirm that AZT can be a recovery therapy against MDR bacteria and thus help clinicians avoid therapeutic impasses.

Finally, in order to anticipate the emergence of resistance in bacteria, some molecules have the significant advantage of not being used alone, of focusing on multiple targets and thus eradicating the infection as quickly as possible.

## Drug Combination Therapy Increases Successful Drug Repurposing

After finding a positive response to a new use, if it does not sufficiently meet the criteria of efficacy, safety, pharmacodynamics, non-toxicity, combination studies with another drug may be considered in order to use it effectively. Drug combinations consist on an association of two or more drugs in order to enhance efficacy of therapeutic strategy and increase chances of clinical applications. It broadens the spectrum of activity of useful antibiotics, for example for serious infections requiring urgent and effective treatment (Zheng et al., 2018). The use of two or more drugs has an impact on different targets, increasing the impairment of microbial function and reducing the risk of resistance emergence (Zheng et al., 2018). The main goal of drug association is to produce a synergistic effect: effect produced by combination is greater than that achieved with any of the drugs used alone. Moreover, if one compound has low activity, another can potentiate and increase it. This reduces the concentration of each individual molecule and can therefore be used at lower doses. This is a real advantage when one knows toxicity of certain drugs (Sun et al., 2016a; Zheng et al., 2018). Sun et al. showed the use of drug combinations reduced toxicity. It increased activity compared to a single therapy when cytotoxicity was proven allowing the use of some drugs in human medicine that were not conceivable on their own (Sun et al., 2016a).

## Drug Repurposing for Combination With Known Antibiotics

Research in drug repurposing for combination with known antibiotics on carbapenem and/or colistin-resistant bacteria has mainly been conducted in association with polymyxins drugs (Table 1). Niclosamide, an anthelmintic drug, known to be active against most tapeworms, seems to interact with the negatively charged outer membrane of colistin-resistant strains leading to a synergistic effect with colistin. This effect was observed on 18 strains with 13 colistin-resistant *A. baumannii* (*pmrB* altered), and 2 colistin-resistant *K. pneumoniae* (*mgrB* and *pmrB* altered) (Ayerbe-Algaba et al., 2018). Colistin combination therapy with selective estrogen receptor modulators (SERM) as tamoxifen, raloxifen and toremifene also exhibited good activity against polymyxin-resistant *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii*. Tested *in-vitro* concentrations could be achievable for human concentration (Hussein et al., 2017; Schneider et al., 2017). In 2019, fluspirilene and Bay 11-7082 have shown promising results by resensitizing a resistant *A. baumannii* to overcome colistin resistance (Cheng et al., 2019). Regarding polymyxin B, synergistic activity with mitotane, an antineoplastic approved for carcinoma treatment, was studied *in-vitro* on 10 strains including carbapenem or polymyxin-resistant GNB. Tests were also carried out on infections of mouse burn wounds, which led to a promising result for the treatment of this type of infection (Tran et al., 2018). Using knowledge of the mechanisms of action, an approach was also tested with some channel-blocking molecules. Indeed, these efflux pump



inhibitors did not have an effect on bacteria alone but combined with an antibiotic, they demonstrated restoration of its activity. The effect is more or less strong but as for neuroleptics (prochlorperazine, chlorpromazine, promazine) associated with meropenem appear to be effective against MDR *A. baumannii* (Yang and Chua, 2013).

Otto et al., showed a potential efficacy of 7 drugs, including 3 antidepressants (amitriptyline, imipramine and sertraline) and 4 antipsychotics (chlorpromazine, clonazepam, haloperidol, and levopromazine) with polymyxin B against 20 tested GNB displaying various resistance mechanisms including carbapenemases (Otto et al., 2019). Only sertraline, chlorpromazine and levopromazine had a synergism effect with polymyxin B against *A. baumannii*, *E. coli* and *K. pneumoniae* isolates. Among all non-antibiotics, only spironolactone, which had only a good efficacy against *E. coli* isolates, showed non-toxic levels of minimum concentration for synergy with polymyxin B (Otto et al., 2019). These findings show that non-antibiotics molecules can be effective in combination but studies need to be pursued to develop association with effective concentrations that are clinically tolerated (Otto et al., 2019).

### Natural Compounds for New Combinations Should Not Be Excluded

Although some natural compounds are not FDA-approved, some may also be part of this process for which a compound is used in another known property. The stilbene and polyphenol resveratrol is produced by various plants (as grapes and blueberries) and is known to have various antioxidant properties and chemopreventive activities. In the bark from *Nauclea pobeguinii*, Cameroonian researchers found this compound and tested it on GNB with MDR phenotypes. It was active alone and in a synergy with streptomycin and ciprofloxacin (Seukep et al., 2016). Rossolini et al. demonstrated in 2018 activity of resveratrol as an antimicrobial agent in combination with colistin on a panel of colistin-resistant (chromosomal or plasmid resistance) GNB (Cannatelli et al., 2018). Resveratrol seems to potentiate colistin activity and thus makes it possible to restore its action among different species and resistance pathways. Thus, another natural compound, pterostilbene, derived from blueberries and grapes and known for its anticancer, anti-inflammatory and antioxidant effects, appears to enhance polymyxin activity *in vitro* and *in vivo* (Zhou et al., 2018). They demonstrated synergistic

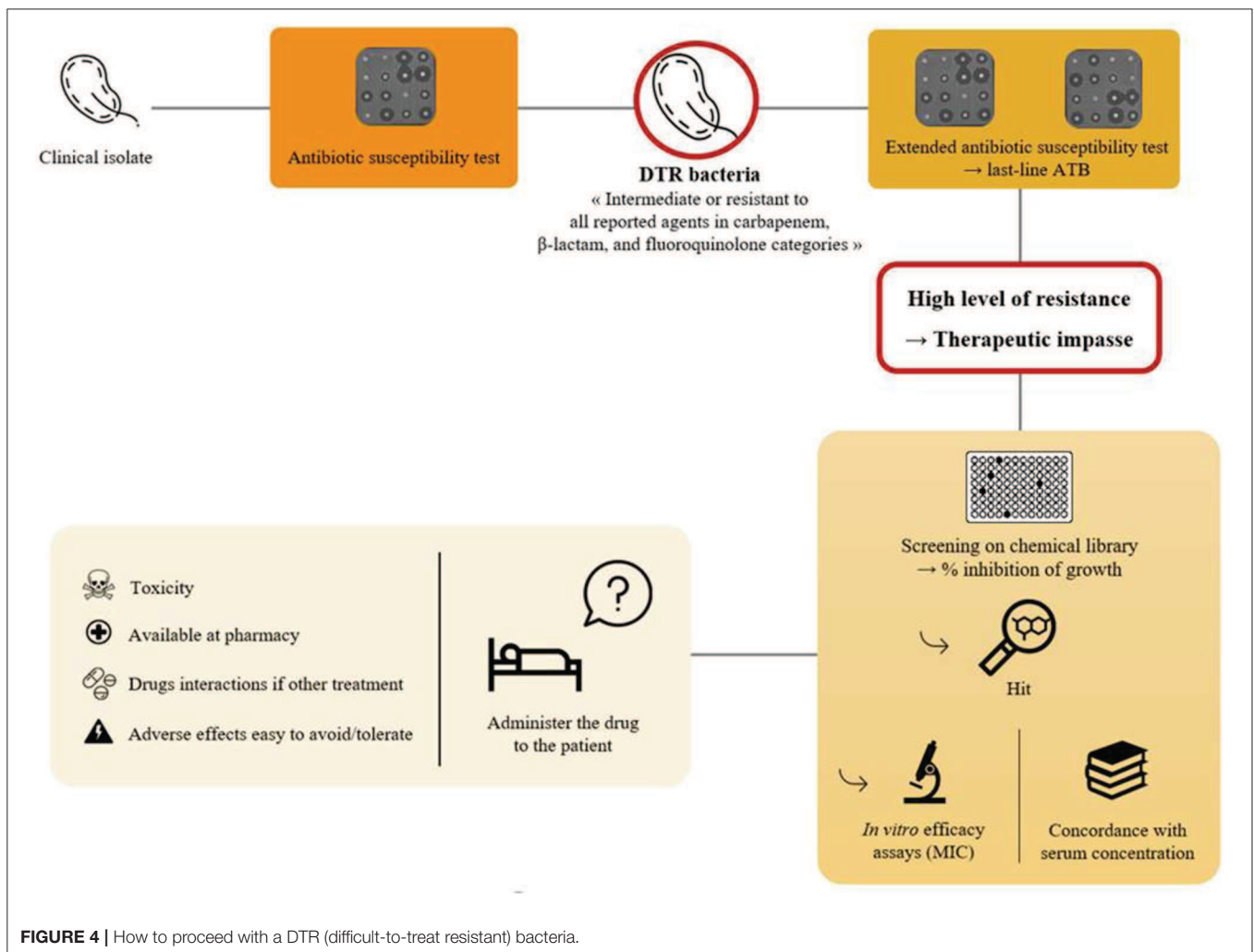


FIGURE 4 | How to proceed with a DTR (difficult-to-treat resistant) bacteria.

effects with colistin and with polymyxin B in *mcr-1* positive strains by 8-fold reducing MIC of polymyxin B with 32 $\mu$ g/ml (Zhou et al., 2018). Pterostilbene could therefore affect *mcr-1* function and restore antibacterial activity of polymyxin B in resistant isolates. Probably safe for human clinical practice and possibly not exerting selective pressure associated with the current ATB, it is therefore considered a good candidate for drug repurposing (Zhou et al., 2018). Lastly, eugenol is a phenylpropanoid present in essential oil of many plants. Thirteen animal *E. coli* strains with colistin resistance were subjected to MIC, time kill and checkerboard assays to evaluate combination between colistin and eugenol (Wang et al., 2018). They observed that *mcr-1* gene expression was down regulated by eugenol and suggested a possible binding between eugenol and MCR-1 protein. (Wang et al., 2018).

All these molecules are still being tested *in vitro* or in clinical trials and none have yet received new indication for MDR infection treatment. However, research on drug repurposing is gaining new dynamism if we can refer to the number of annual publications in recent years (Figure 3).

## PERSPECTIVES

In conclusion, this review therefore addresses two main aspects, both the emerging drug repurposing strategy and resistance to last-line antibiotics, carbapenems, and colistin. A new economic model is to be considered for antibiotic development because industries do not seem interested in this new strategy (Zheng et al., 2018). Indeed, as antibiotics are not part of chronic treatment strategies, this could not be as economically attractive (Conly and Johnston, 2005). This disinterest in antibiotics research is reflected in their absence in programs of future developments of major pharmaceutical companies (Spellberg et al., 2004). Start-ups or small companies, on the other hand, can see an interest in taking back antibiotics that have failed in clinical phases, for example. They believe they have different drug development strategies and do not require as many benefits to cover their costs compared to multinational pharmaceutical companies (Fernandes and Martens, 2017). However, Phase 3 clinical trials for new and repurposed drugs remain very expensive: it is estimated between \$40 million and \$300 million of USD (Azvolinsky, 2017). With such a budget, this does not work in favor of small firms.

As for the question of how to treat bacterial resistance, only one answer has not been found and the future offers us new possibilities. Various strategies are being considered as treatment using fecal microbiota (Davido et al., 2017), antimicrobial peptides (Hashemi et al., 2017) or bacteriophages (Parmar et al., 2017). A *Streptomyces sp.* present in alkaline soil in Ireland has been in the spotlight recently to inhibit growth of MDR bacteria (Terra et al., 2018) which reminds of Flemming discovery. Additionally, the discovery and studies on the CRISPR/Case9 system may suggest that it may be the ultimate weapon to fight infectious diseases and thus control antibiotic resistance (Doerflinger et al., 2017).

Question of drug repurposing remains rather wide. Although this seems to be a better solution, drug combinations can also lead to adverse interactions. First, toxic side effects can be increased. Then, concerning compound galenic, physico-chemical interactions and differences in stability, solubility and conservation can result from the combination of two molecules making it incompatible. Formulation then becomes more complicated (Sun et al., 2016a). On the other hand, we must change this vision where each drug belongs to only one box. Clinicians may have difficulty understanding why a biologist recommends the use of an anti-cancer or anti-inflammatory drug to treat their cystic fibrosis patient's bacteremia rather than a last resort antibiotic they have always used. Communication in this sense remains essential between health professionals and clinical studies to prove these activities are critical. However, with current knowledge on drug repurposing as antibacterial agents and the problematic to find an alternative therapeutic in some situations, screening of non-antibiotics in a "à la carte" way can be an issue (Figure 4). In the area of personalized medicine, we could imagine a personalized antibiotic susceptibility testing in case of infection caused by a highly resistant bacterium. If all last-line antibiotics have been tested and appear insufficient to successfully treat the patient, testing non-antibiotic drugs that are potentially active on the pathogen, alone or in combination with antibiotics, could help clinicians use these drugs. As previously reported by Kadri et al., these difficult-to-threat bacteria refer to bacteria that are resistant to all first-line antibiotics. It represents less than 1% of isolates and those that are resistant to second-line antibiotics are even rare (Kadri et al., 2018). This solution, combined with monitoring of serum levels and adverse events such as dialysis of a nephrotoxic drug (Rolain and Baquero, 2016), could offer great potential for treating a patient with this MDR bacterium. The problem remains to be able to routinely test a large panel of molecules, in an automated, reproducible, and not too expensive way.

## AUTHOR CONTRIBUTIONS

LP, SB, and J-MR drafted and revised the manuscript. All authors read and approved the final manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## IV. Criblage de molécules médicamenteuses sur des bactéries multi-résistantes

Cette seconde partie se concentre sur la mise en pratique du repositionnement moléculaire : son application est envisagée pour une alternative de traitement sur des bactéries considérées comme multi-résistantes (BMR).

La résistance aux antibiotiques est largement distribuée et peut toucher toutes les espèces rencontrées en pratique clinique<sup>38</sup>. A l'hôpital, certaines infections bactériennes représentent de vrais défis pour les cliniciens. Ces bactéries sont qualifiées de « difficile à traiter » car elles sont résistantes aux agents de première ligne<sup>25</sup>. Par exemple, les patients greffés pulmonaires atteints de mucoviscidose peuvent être infectés par des bactéries opportunistes, ayant des résistances acquises à de nombreux antibiotiques<sup>39</sup>. Ces bactéries restent des cas isolés mais peu de solutions restent à disposition. Cette stratégie peut donc aider à contourner ce phénomène de résistance et apporter une réponse rapide aux cliniciens sur des molécules efficaces à administrer.

De ce fait, dans l'**Article 2** « *In vitro screening of multidrug-resistant and extensively drug-resistant bacteria on a 1,280 FDA-approved drugs library* », nous avons mis en place ce criblage par une méthode phénotypique. Nous avons étudié l'efficacité in vitro de 1280 molécules sur la croissance bactérienne d'une collection de BMR cliniques. Nous avons sélectionné un panel de différentes bactéries présentant des phénotypes de résistances variés puis nous avons étudié les différents composés efficaces au terme de notre essai. La mise en application clinique étant le but de cette méthode, nous avons regardé si les concentrations sériques chez l'homme de ces hits pouvaient être compatibles avec la concentration testée ou si la voie d'administration était adaptée à un usage immédiat. Certains anciens antibiotiques, des molécules candidates pour un possible repositionnement moléculaire ou des associations de molécules présentent un espoir pour le traitement de ces BMR, les BGN non fermentaires restant des bactéries pour lesquelles il existe le moins de solutions thérapeutiques. En revanche, nous avons conclu que cette technique devait être appliquée au cas par cas.

L'**Article 3** « *Pandoraesa nosoerga, drug repurposing and whole genome sequencing in a lung recipient* » est un *case report* représentant justement un exemple de situation clinique d'infection à bactérie « difficile à traiter » face auquel le clinicien peut être confronté. Il s'agit d'une patiente atteinte de mucoviscidose, colonisée chronique à *P. nosoerga*. En 2018, cette patiente avait subi une greffe bi-pulmonaire et hépatique face à l'état avancé de sa mucoviscidose. Malgré une prophylaxie antibiotique, elle redéveloppa une infection à *P. nosoerga* XDR en post-opératoire. Deux questions ont été posées face à ce cas : i) le recours à un traitement antibiotique efficace : le repositionnement moléculaire a été utilisé

au laboratoire dans ce cas pour trouver une option thérapeutique car les traitements antibiotiques utilisés en clinique n'étaient pas efficaces ; ii) la source de la recolonisation chez cette patiente : en effet, son frère, atteint également de mucoviscidose était également colonisé à *P. nosoerga*. Nous avons ainsi réalisé une analyse comparative génomique de différentes souches pré- et post-greffe de la patiente, ainsi qu'une souche de son frère, pour comprendre s'il s'agissait d'une infection *de novo* ou d'une réinfection à partir de la souche initiale.

## **1. Article 2. *In vitro* screening of multidrug-resistant and extensively drug-resistant bacteria on a 1,280 FDA-approved drugs library - Peyclit L., Baron S., et Rolain J-M.**

En cours de rédaction

Le repositionnement des médicaments est considéré comme une stratégie alternative pour offrir aux cliniciens des médicaments rapidement disponibles et pour lesquels les éventuels effets toxiques sont connus et peuvent être donc contrôlés. Ici, nous avons rassemblé une collection de BMR isolées dans notre hôpital à Marseille et effectué un criblage phénotypique sur une bibliothèque de 1280 médicaments approuvés par la FDA. Nous avons choisi cette bibliothèque, de fabrication française, car elle représentait une grande diversité chimique et pharmacologique. Les molécules étaient hors brevet et la majorité étaient approuvées par des agences de santé comme la FDA ou European Medicines Agency (EMA).

Nous avons collecté 2 cocci à GP (*E. faecium* P5014, *S. aureus* P1943) et 6 BGN (*A. baumannii* P1887, *K. pneumoniae* P9495, *P. aeruginosa* P6540, *B. multivorans* P6539, *P. nosoerga* P8103 et la souche de référence *E. coli* DSM 105182). La sélection de ce panel a été faite d'une part pour avoir un panel représentatif des bactéries les plus fréquemment responsables d'infections multi-résistantes (*S. aureus*, *E. faecium*, *K. pneumoniae*, *A. baumannii*, *E. coli*), présentant des mécanismes de résistance fréquents aux thérapies de dernière ligne comme la colistine ou les carbapénèmes (comme les gènes *mcr-1*, *NDM-1*, *OXA-48*) et d'autre part par des BMR qui ont posées de vrais problèmes thérapeutiques en situation clinique (*P. nosoerga*, *B. multivorans* et *P. aeruginosa*).

Au total, après un screening à 10 µmol/l, 107 composés parmi 9 classes thérapeutiques ont inhibé plus de 90 % de la croissance de nos BGN et cocci à GP; 7,5 % étaient des médicaments oncologiques. Des composés non anti-infectieux ont été répertoriés, comme l'azapérone et le lomérizine efficaces contre *E. coli*, la déféroxamine contre *P. nosoerga* ou la dronédarone, l'auranofine, le 5-fluorouracile contre les cocci à GP. La chlorhexidine, un antiseptique, constituait le seul hit commun à tout notre panel. Par la

suite, nous avons comparé nos résultats avec les données pharmacocinétiques de la littérature. Le déféroxamine associée à l'acide ascorbique pourrait traiter les infections à *P. nosoerga* ou l'auranofine associée à la colistine pour la souche XDR de *P. aeruginosa*. L'utilisation d'antibiotiques à des concentrations supérieures ou en association restent également des solutions à envisager. Celles-ci doivent être étudiées au cas par cas en amont pour vérifier leur efficacité antibactérienne, comprendre leurs mécanismes d'action et confirmer leur pertinence pour une administration adéquate.

Le repositionnement moléculaire est une méthode rapide et facile à réaliser, permettant de trouver des molécules efficaces sur des bactéries spécifiques, difficiles à traiter pour les cliniciens. Certains de nos résultats représentent alors un premier pas vers l'utilisation de cette stratégie de repositionnement des médicaments pour traiter les infections à BMR.



1 **Title:** *In vitro* screening of multidrug-resistant and extensively drug-resistant bacteria on a  
2 1,280 FDA-approved drugs library.

3 **Running title:** High throughput drug screening on MDR/XDR bacteria

4 **Keywords:** multidrug-resistant bacteria, extensively-drug resistant bacteria, alternative  
5 strategy, drug repurposing, old drugs, antibiotic combination

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21 **Abstract**

22 Drug repurposing is considered an alternative strategy against multidrug-resistant (MDR)  
23 bacterial to provide clinicians with rapidly available and marketed drugs. **Methods:** Here, we  
24 gathered a collection of MDR bacteria from our hospital and performed a phenotypic high-  
25 throughput screening with a 1,280 FDA-approved drug library. We compared our results with  
26 pharmacokinetic data from the literature. **Results:** We collected 2 Gram-positive (GP)  
27 (*Enterococcus faecium* P5014, *Staphylococcus aureus* P1943), 6 Gram-negative (GN)  
28 (*Acinetobacter baumannii* P1887, *Klebsiella pneumoniae* P9495, *Pseudomonas aeruginosa*  
29 P6540, *Burkholderia multivorans* P6539, *Pandoraea nosoerga* P8103 and the reference strain  
30 *Escherichia coli* DSM105182). Our MDR panel carried resistance genes or displayed  
31 phenotypic resistance to last-line therapies like carbapenems, vancomycin or colistin. A total  
32 of 107 compounds from 9 therapeutic classes inhibited >90% growth of our GN and GP  
33 bacteria, 7.5% were oncology drugs. The common hit was the antiseptic chlorhexidine.  
34 Activity of niclosamide, carmofur and auranofin was confirmed on our methicillin-resistant *S.*  
35 *aureus*. Zidovudine was effective against our colistin-resistant *E. coli* and carbapenem-  
36 resistant *K. pneumoniae*. Trifluridine, an antiviral, was effective against *E. faecium*.  
37 Deferoxamine mesylate inhibited the growth of XDR *P. nosoerga*. Despite extensively drug-  
38 resistant non-fermenting GN displayed less hits, increasing concentrations or using some  
39 combination therapies can be an alternative solution to treat these infections. **Conclusion:**  
40 Drug repurposing by an *in vitro* screening of a drug library is a rapid and easy method for  
41 clinicians to find an effective drug on specific bacteria. These results represent a first step to  
42 use a drug repurposing strategy to treat MDR bacterial infections.

43

44

45

## 46 **Introduction**

47 Emergence and spread of multidrug-resistant (MDR) pathogens represent a global healthcare  
48 question nowadays. Treating infections caused by MDR bacteria can be challenging for  
49 clinicians<sup>1,2</sup>.

50 Multi-drug resistance is conventionally defined as resistance to  $\geq 1$  agents of three or more  
51 antibiotic class<sup>3</sup>. They can also be qualified as extensively drug-resistant (XDR) if they  
52 remain susceptible to only one or two antimicrobial categories or pandrug-resistant (PDR) if  
53 they are resistant to all of antimicrobial categories<sup>3</sup>. Definition of “difficult-to-treat” (DTR)  
54 bacteria seems to be more suitable for clinical practice, qualifying bacteria when they are  
55 resistant to all the first-line antibiotics as  $\beta$ -lactams or fluoroquinolones<sup>4</sup>. In any case,  
56 treatment of MDR bacterial infections can be limited due to resistance to first-line antibiotics  
57 and now, to last-line therapies<sup>5</sup>. Resistant Gram-positive (GP) cocci showed resistance to  
58 glycopeptides<sup>6</sup>. Carbapenems or polymyxins are not efficient against Gram-negative (GN)  
59 strains, because of carbapenemase enzymes or structural modifications<sup>6</sup>. Therapeutic impasses  
60 due to resistant bacteria remain infrequent, but in some situations, clinicians must now tackle  
61 with resistance to treat fragile patients<sup>5</sup>. Strategies to prevent therapeutic impasses are  
62 essential, especially for non-fermenting GN bacteria. Indeed, they display a broad intrinsic  
63 resistance and are often infectious agents in immunosuppressed patients<sup>7</sup>. As a result, many  
64 alternative strategies are now considered to enhance the treatment of infectious diseases by  
65 MDR bacteria, such as phage therapy or drug repurposing<sup>8</sup>.

66 Drug repurposing objective is to identify a novel clinical use for an existing drug approved in  
67 clinical medicine for a different indication. This method should be considered for treating new  
68 pathogens or agents for which no effective treatment is available<sup>9</sup>. Indeed, current  
69 pharmaceutical companies have no economic interest in developing new molecules to treat  
70 MDR bacteria that remain isolated cases<sup>9</sup>. As the situation is a public health issue<sup>10</sup>, find

71 effective drugs already in our pharmacies can be an excellent alternative. Plus, it offers  
72 various advantages such as an economic drug development or an acceleration of the process  
73 by skipping preclinical trials<sup>11</sup>. To treat MDR infections, repurposing a drug seems to be an  
74 easy and rapid strategy. One of the method is phenotypic-based assay and consists of testing  
75 *in vitro* the efficacy of a large quantity of drugs on the survival of a bacterium, as a high-  
76 throughput screening<sup>9</sup>. Using this strategy, some efficient drugs were found so far as ribavirin  
77 against *Candida* strains<sup>12</sup>, the antiretroviral zidovudine against Enterobacteriaceae<sup>13</sup> or the  
78 antihelminthic niclosamide against *Staphylococcus aureus* biofilms<sup>14</sup>.

79 Research are mainly focusing on finding a way to fight against bacteria belonging to the  
80 ESKAPE group (including *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*,  
81 *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.*), but some  
82 “intrinsically” multi-drug resistant bacteria constitute major treatment challenges. This is  
83 especially the case in cystic fibrosis (CF) patients, particularly after pulmonary transplant  
84 when post-transplantation immunosuppressive therapy increases the risk for opportunistic  
85 infections<sup>15</sup>, including non-tuberculous mycobacteria<sup>15</sup> or non-fermenting GN bacterial<sup>16</sup>  
86 infections. In the presence of immunosuppression, the impact of PDR bacterial pathogens  
87 contributes to greater mortality in the CF population because of treatment difficulties<sup>17,19</sup>.

88 The objective of our study was to decipher which drugs may still be effective against bacteria  
89 that have a high level of drug resistance and pose potential treatment problems. We focused  
90 on various MDR or XDR bacteria, especially those found in CF patients. We investigated *in*  
91 *vitro* efficacy of drugs with unknown antibacterial potential that could be a first step towards a  
92 new drug repurposing strategy. Here, we gathered a collection of various drug-resistant  
93 bacteria of our hospital and performed a phenotypic high-throughput screening with a 1,280  
94 FDA-approved drug library.

95

## 96 **Methods**

### 97 **Collection and susceptibility of bacterial strains**

98 With the exception of the *mcr-1* reference strain *Escherichia coli* (DSM 105182), all strains  
99 (*E. faecium* P5014, *S. aureus* P1943, *Acinetobacter baumannii* P1887, *K. pneumoniae* P9495,  
100 *P. aeruginosa* P6540, *Burkholderia multivorans* P6539 and *Pandoraea nosoerga* P8103) have  
101 been collected for their antibiotic resistance profile from clinical samples. To determine  
102 antibiotic susceptibility, the standard disk diffusion method on Mueller-Hinton agar  
103 (BioMérieux, Marcy l'Etoile, France) and breakpoints assessment were performed according  
104 to European Committee of Antimicrobial Susceptibility Testing (EUCAST)  
105 recommendations. When breakpoints were not available in EUCAST, we used  
106 recommendations from the French Society of Microbiology (CA-SFM). We used the  
107 previously published definitions from the study of A. Magiorakos<sup>3</sup> to categorize MDR  
108 (acquired non-susceptibility to at least one agent in three or more antimicrobial categories)  
109 and XDR (non-susceptibility to at least one agent in all but two or fewer antimicrobial  
110 categories) phenotypes.

### 111 **Preparation of isolates**

112 The isolates were inoculated on TSA medium (Trypticase soy agar, BioMérieux) at 37°C for  
113 18-24 hours. Strains were suspended at 0.5 MacFarland ( $10^8$  CFU/ml) in 0.9% NaCl and  
114 diluted 1/100 ( $10^6$  CFU/ml) in cation adjusted Mueller-Hinton broth media (CAMHB, Merck  
115 KGaA, Darmstadt, Germany).

### 116 **High-throughput screening assay**

117 The screening test was performed on 96-well microplates with 1,280 FDA-approved drugs  
118 from a chemical library (Prestwick-Chemical, Illkirch Graffenstaden, France). Regardless of  
119 the protocol, the final drug concentration in each well was always 10  $\mu$ mol/l. Dilution of the

120 initial plates was performed to achieve a DMSO concentration of less than 0.1% in all final  
121 plates.

122 Plates were prepared in duplicates with 80  $\mu$ L of CAMHB, 10 $\mu$ L of drugs at 100 $\mu$ mol/l and  
123 10 $\mu$ L of each bacterium at 10<sup>6</sup> CFU/ml and with negative and positive controls, incubated at  
124 37°C for 18-24 hours shaking at 300 rpm.

125 Bacterial growth was measured by spectrophotometry (Multiskan Spectrum, Thermo Fisher  
126 Scientific, Waltham, Massachusetts, United States). Then, bacterial growth inhibition rate was  
127 calculated for each molecule/bacterium with the following equation as a function of  
128 absorbance (A) in the wells:

$$129 \quad \text{Bacterial growth inhibition (\%)} = \left[ 1 - \left( \frac{A_{\text{sample}} - A_{\text{negative control}}}{A_{\text{positive control}} - A_{\text{negative control}}} \right) \right] \times 100$$

130 Hits were selected when growth inhibition rate is greater than 90%.

131

## 132 **Results**

### 133 **Antibiotic resistance profile and genomic support of our strains panel**

134 The two MDR GP strains were a clinical strain of methicillin- and glycopeptides- resistant *S.*  
135 *aureus* P1943 carrying the *mecA* gene and a clinical strain of vancomycin-resistance *E.*

136 *faecium* P5015 carrying a *vanA* gene (**Table 1**). Among the 6 GN bacteria tested, 3 isolates

137 (*E. coli* DSM 105182, *K. pneumoniae* P9495 and *A. baumannii* P1887) were classified as

138 MDR and 3 isolates were classified as XDR (*P. aeruginosa* P6540, *B. multivorans* P6539 and

139 *P. nosoerga* P8103) as shown in **Table 1**. Four isolates were carbapenem-resistant (P9495,

140 P1887, P6539, P8103) including three producing NDM-1 for *A. baumannii*, OXA-48 for *K.*

141 *pneumoniae* and OXA-158 for *P. nosoerga*. Five of them were resistant to colistin, a last-line

142 therapeutic drug (all except *A. baumannii*). The *E. coli* strain carried a plasmid-mediated

143 colistin resistance gene, *mcr-1* (**Table 1**).

### 144 **General high-throughput screening results**

145 Various hits with growth inhibition rate of 90% or more were found as shown in **Figure 1** and  
146 non-anti-infective compounds were listed for each bacterium in **Table 2**.

147 Overall, 62 hits inhibited at least 90% of the *S. aureus* growth, 37 for *E. faecium*, 27 for *E.*  
148 *coli*, 16 for *K. pneumoniae* and 16 for *A. baumannii* (**Figure 1**). About the XDR bacteria, 6  
149 hits were found for *P. aeruginosa*, 9 for *P. nosoerga* and 15 for *B. multivorans*.

150 Overall, of the 9 therapeutic classes involved, 107 different hits were found in total including  
151 20 (19%) not belonging to the infectiology class (**Figure 1**). Among that, 8 (7.5%) oncology  
152 drugs were found active on GP bacteria: tamoxifen citrate, gemcitabine, carmofur,  
153 floxuridine, pemetrexed disodium, raltitrexed, 5-fluorouracil and methotrexate (**Table 2**). For  
154 the two MDR GP bacteria tested, 23 common hits were noticed, including an antiarrhythmic,  
155 two antiseptics, twelve antibacterials, an antiprotozoal, an antihelminthic, an antirheumatic,  
156 two antifungal, and three antineoplastics (**Figure 2**). GN bacterial hits all belonged to  
157 infectiology class, except two (azaperone and lomerizine hydrochloride) for *E. coli* and  
158 deferoxamine mesylate for *P. nosoerga* (**Figure 1, Table 2**). Between GN and GP bacteria,  
159 the antiseptic chlorhexidine was the only common hit among all the therapeutic class (**Figure**  
160 **2**).

#### 161 **Main hits by species**

162 All hits were reported in **Figure 2**, but we mentioned below those that required our attention  
163 for each species.

#### 164 - *S. aureus*:

165 Various antibiotics (furazolidone, monensin) and antiseptics were effective while  
166 antihelminthics such as closantel, niclosamide or pyrvinium pamoate or antifungals as  
167 sulconazole inhibited *S. aureus*. The antiarrhythmic dronedarone was effective against our  
168 strain (**Table 2**).

#### 169 - *E. faecium*:



170 Among the compounds, we noticed that antifungal azoles were effective against *E. faecium*  
171 (butoconazole, econazole, oxiconazole, tioconazole, sulconazole). All those drugs were  
172 intended for topical use. Amiodarone, methotrexate and an antiviral drug, trifluridine, were  
173 also found effective (**Table 2**). Clomiphene citrate and tamoxifen, selective oestrogen  
174 receptor modulators, inhibited the growth of our resistant *E. faecium*.

175 - ***E. coli***:

176 *E. coli* bacterial growth was inhibited by anti-infective drugs such as known antibiotics or  
177 antiseptics, but also by an antiviral: zidovudine. Azaperone and lomerizine hydrochloride  
178 (Central Nervous System, CNS) were two non-anti-infective drugs effective against *E. coli*  
179 (**Table 2**).

180 - ***K. pneumoniae***:

181 Antibiotics as tetracyclines or aminoglycosides and the antiviral zidovudine inhibited the  
182 growth of *K. pneumoniae*.

183 - ***A. baumannii***:

184 Drugs not belonging to the anti-infectious class have shown very limited efficacy in inhibiting  
185 the growth of *A. baumannii*. As anti-infectious drugs, fluroquinolones, rifamycin and  
186 antiseptics (merbromin, triclosan and chlorhexidine) were found to be the most effective  
187 against our carbapenem-resistant *A. baumannii*. Colistin was also effective at 11,5 mg/l (10  
188  $\mu\text{mol/l}$ ).

189 - **XDR non-fermenting GN**:

190 Against our XDR bacteria, fewer drugs were effective, and they were mostly antiseptics or  
191 antibiotics. However, this screening may point to potential combination or higher dosage  
192 regimens on drugs that are still effective against XDR strains (**Table 3**). For instance, higher  
193 concentrations of colistin were effective against *P. aeruginosa* when it was initially resistant  
194 in our routine tests (**Table 2**). Combination of drugs among old drugs, broad spectrum



195 antibiotics or non-anti-infective compounds may provide killing effects<sup>20</sup>. Rifampicin and  
196 minocycline were both effective on *P. nosoerga*, so MIC and checkerboards of the  
197 combination should be performed for that strain as it already showed good results<sup>21</sup>. In terms  
198 of drug repurposing, by reducing our detection limit to 55%, we were able to find an anti-  
199 rheumatic agent: auranofin was effective against *P. aeruginosa*. Deferoxamine mesylate was  
200 the only non-anti-infective hit against that *P. nosoerga* (**Table 2**).

### 201 **Comparison with data from the literature**

202 We compared our results with pharmacokinetic data from literature studies for each bacterium  
203 in order to repurpose our hits (**Annex 1**).

204 Concentration tested for the antiarrhythmic dronedarone in the screening was effective against  
205 *S. aureus*, but is much higher than the plasma concentrations found in humans<sup>22</sup>. However,  
206 amiodarone was active at achievable concentration on *E. faecium*. This antibacterial effect on  
207 GP bacteria has not been demonstrated to our knowledge. The narrow therapeutic window of  
208 antiarrhythmics and their side-effects lead to a challenging clinical use and can be  
209 controversial in that case<sup>23</sup>.

210 Anticancer drugs have also shown some effectiveness against GP bacteria. In our screening,  
211 gemcitabine was effective at 2.6 mg/l for *S. aureus* and *E. faecium*. In the literature, the  
212 maximum serum concentration (C<sub>max</sub>) could be 2 mg/l with infusion at 600 mg/m<sup>224</sup> or 40.9  
213 mg/l with an administration of 2000mg/m<sup>225</sup>. Similarly, infusion of 500 mg/m<sup>2</sup> of pemetrexed  
214 leading to a C<sub>max</sub> of 119 mg/l in adult cancer patients could inhibit the growth of *E. faecium*  
215 as in our assay (tested at 4,7mg/l)<sup>26</sup>.

216 Previous pharmacokinetics data on deferoxamine mesylate revealed a steady-state  
217 concentration of 7 µmol/l (3.9 mg/l) using continuous intravenous deferoxamine infusion at  
218 50 mg/kg/d<sup>27</sup> which is in the same range as our screening concentration.

219

## 220 Discussion

### 221 **What are the potential therapeutic options for treating these MDR bacteria?**

222 After analyzing of results of our screening of 1,280 molecules on a panel of XDR or MDR  
223 strains, we found several possible options to help fight these bacteria.

224

#### 225 - **To find a common hit**

226 The only common hit we could find among 1280 drugs for this panel was chlorhexidine. By  
227 disrupting the cell membrane and interfering with osmosis<sup>28</sup>, this widely antiseptic and  
228 disinfectant is used, for instance, as a topical agent for skin decolonization or to sterilize  
229 surgical tools<sup>29</sup>. After all, this common result is in favour of the chlorhexidine bathing  
230 recommended before performing a transplant to limit GN bacterial infections<sup>30,31</sup>. Its efficacy  
231 among MDR and XDR bacteria is also supported by previous studies on VRE<sup>32</sup>, MRSA<sup>33</sup>,  
232 carbapenemase-producing *K. pneumoniae*<sup>34</sup> or MDR GN<sup>31,35</sup>. Despite its wide use in  
233 prevention, its effectiveness must be monitored for the development of resistance or adverse  
234 effects<sup>36</sup>. Anyway, not finding a common hit for these bacteria for systemic use is not  
235 worrisome as this would also affect the vital microbiota.

236

#### 237 - **To repurpose a molecule**

238 In our study, some hits were specifically efficient against bacteria and were not initially  
239 marketed for that use: they can be tied to drug repurposing<sup>9</sup>. In that way, the antihelminthic  
240 niclosamide, with more than 90% of inhibition of *S. aureus*, has already been widely proposed  
241 to treat drug-resistant bacteria<sup>14,37-39</sup>. Its antibacterial potential has been described for hospital  
242 acquired infections as MRSA<sup>14</sup> or intestinal decolonization of VRE<sup>39</sup>, but also in combination  
243 with colistin to treat colistin-resistant GN bacilli infections<sup>37,38</sup>. As carmofur and auranofin are  
244 also effective on MRSA, they have been further investigated for their anti-biofilm activity<sup>40</sup>.

245 Efficacy of selective oestrogen receptor modulators on *E. faecium* confirmed the idea of  
246 repurposing some oestrogen receptor antagonists to treat infectious diseases<sup>41</sup>. In addition, the  
247 antimetabolite methotrexate was effective against the growth of *E. faecium* and could act by  
248 inhibiting dihydrofolate reductase such as trimethoprim, which was also effective. An old  
249 study conducted in 1974 reported methotrexate activity with a MIC of 0.15 µg/l against  
250 *Streptococcus faecium*<sup>42</sup>, previous name of *E. faecium*, confirmed on *S. faecalis* ten years  
251 after<sup>43</sup>. Antibacterial effect of zidovudine on *E. coli* and *K. pneumoniae*, previously shown in  
252 1987<sup>44</sup>, confirmed our report about its potential use to treat infections on colistin-resistant GN  
253 bacteria<sup>45</sup>.

254 However, drug repurposing is not feasible for all cases. Concerning *E. coli*, the veterinary use  
255 of azaperone<sup>46</sup> and the low serum concentration for lomerizine in humans<sup>47</sup> do not allow these  
256 CNS drugs for an immediate clinical use but require further investigations (**Annex 1**).

257 Paromomycin, effective against *K. pneumoniae*, is usually used to treat parasite infections  
258 such as amebiasis. Nevertheless, like neomycin, these aminoglycosides display a poor  
259 intestinal absorption which is not suitable for systemic infections. Paromomycin can still be  
260 used for intestinal decolonisation of resistant Enterobacteriaceae<sup>48</sup>.

261

#### 262 - Using drug associations

263 Combination therapy is recommended for MDR bacteria treatment<sup>9</sup>. It allows clinicians to use  
264 drugs at lower concentrations than in monotherapy through a synergistic effect, as shown by  
265 Lee J. H. *et al.* with rifampicin and colistin against MDR *A. baumannii*<sup>49</sup>. Several of our  
266 results could be used in this strategy, after verifying the effectiveness of the combination with  
267 a checkerboard assay. Fluoroquinolones in combination with β-lactams are promising, as  
268 shown by *in vitro* testing against *Burkholderia cepacia*<sup>50</sup> or evaluation in a large cohort study  
269 against GN bacilli bacteraemia<sup>51</sup>. *In vitro* studies reported a good efficiency of imipenem in

270 combination with rifampicin or colistin against MDR clinical isolates of *P. aeruginosa*<sup>52,53</sup>.  
271 Furthermore, auranofin, which was effective against our XDR *P. aeruginosa*, has been  
272 studied in combination with polymyxins on GN species that permeabilize the outer membrane  
273 of GN and help auranofin enter the bacteria cell. The combination with colistin on *P.*  
274 *aeruginosa* showed a synergistic effect<sup>54</sup> and addition of ceftazidime inhibited > 80% growth  
275 of 10 MDR, including *P. aeruginosa*<sup>55</sup>.  
276 Trifluridine, an antiviral molecule is used in monotherapy as anti-herpesvirus eye drops. It has  
277 low bioavailability after clinical administration as it is rapidly degraded via thymidine  
278 phosphorylase. Therefore, a combination with a potent thymidine phosphorylase inhibitor,  
279 tipiracil is now studied as an antineoplastic use<sup>56</sup>. This combination could be tested on  
280 resistant *Enterococcus sp.* against systemic infections.  
281 Deferoxamine mesylate is used to treat iron overdose as hemochromatosis because it binds  
282 iron and aluminium. This drug effective against *P. nosoerga* is not known to date to be used  
283 as antibacterial monotherapy. By complexing with aluminium or gallium, deferoxamine acted  
284 with a Trojan horse effect, carrying these toxic metal ions into the bacterial metabolism<sup>57</sup>.  
285 Moreover, using with ascorbic acid, deferoxamine showed a bacterial growth inhibiting  
286 effect<sup>58</sup> and added to gentamicin, this triple combination displayed synergistic effect against  
287 some *E. coli* strains<sup>59</sup>. Easy to apply, this association should be assessed by performing MIC  
288 assays on our strains.

289

#### 290 - **Damaging basic mechanisms of bacteria**

291 Although using old drugs or combinations can be successful, drugs acting on essential  
292 mechanisms as DNA or protein cycle of all bacteria are frequently represented.  
293 In addition, our screening concentration was for 4 oncology molecules in the human  
294 concentration range (**Annex 1**). Some activities have been already reported like 5-

295 fluorouracil, tamoxifen, floxuridine, pemetrexed disodium or gemcitabine in GP or in  
296 resistant GN strains<sup>9,60,61</sup>. A study testing tamoxifen in combination with polymyxins against  
297 XDR GN pathogens used achievable concentration for oral administration<sup>61</sup>. However, we are  
298 aware that anti-cancer drugs would not only target bacteria but would also regulate immunity  
299 and host response<sup>62</sup>. Since these drugs can disrupt the immune response, it is doubtful whether  
300 their use to fight infectious diseases would be beneficial. In a review of Soo V. *et al.*,  
301 similarities between cancer cells and bacterial infections encouraged the use of those agents  
302 with a broad activity, which acts on cell cycle<sup>63</sup>. Society might be frightened to give such  
303 therapies due to many side effects to treat infectious diseases. However, given our results,  
304 using anticancer drugs might be the only solution to treat patients infected with a XDR  
305 bacterium for which no treatment remain available<sup>64</sup>.

306 On the other hand, fluoroquinolones and tetracyclines were effective in most of our panel.  
307 Those antibiotics have a broad spectrum, with activity on GP and GN bacteria. Cyclins inhibit  
308 protein synthesis and fluoroquinolones prevent from DNA replication and transcription: those  
309 mechanisms are essential for a bacteria life and therefore highly conserved in all species.  
310 However, bacteria can adapt quickly to these antibiotics developing several resistance  
311 mechanisms, leading to cross-resistance and increasing MIC<sup>65</sup>. Our *B. multivorans* was  
312 previously ciprofloxacin-resistant with the disk diffusion, but became susceptible in the  
313 screening test, where the concentration tested was 3.31 mg/l (10 µmol/l). This difference is  
314 due to the concentration used in the screening method being higher than the usual dose used  
315 in sensitivity testing and medical practice. The use of a higher concentration of ciprofloxacin  
316 or other fluoroquinolones could treat *B. multivorans* infection (**Table 3**). This is in accordance  
317 with previous successful studies with an increase of dosage regiments against PDR bacteria<sup>66</sup>,  
318 leading to a better clinical response and circumvent resistance<sup>64</sup>.

319 Finally, few other anti-infective agents were effective pointing essential mechanisms.  
320 Antiviral nucleosides analogues like trifluridine (*E. faecium*) or zidovudine (against  
321 Enterobacteriaceae<sup>13</sup>) target the inhibition of DNA synthesis, which can explain their effect on  
322 bacterial inhibition growth. In addition to having some synergy with antibiotics, nucleosides  
323 analogues should be considered as new weapons against bacterial infection, as suggested by  
324 A. E. J. Yssel et al<sup>60,67</sup>.

325

### 326 - **Personalizing the treatment**

327 This *in vitro* screening method test a great number of approved drugs with a high and fixed  
328 concentration in a short time for one isolate. Microdilution assays then verify the MIC: this  
329 allows an estimate of whether the required concentration to be achieved in plasma is reached  
330 by using the molecule at the doses approved in the PK/PD studies. Beyond the phenotypic  
331 method, data from innovative tools and sequencing could be compared with *in silico*  
332 databases of molecules<sup>68,69</sup>. In a future perspective, we could imagine a personalized  
333 medicine, testing isolated DTR bacteria to find the most efficient drug<sup>7</sup>. For instance, this  
334 could be the solution for opportunistic bacteria that have many resistance determinants such  
335 as Burkholderiales<sup>70</sup>. The clinical impact of this screening can be significant: as these  
336 molecules are already approved and marketed, they can be quickly made available to  
337 clinicians. The risk-benefit of patients facing such infections must be assessed, as well as the  
338 management of certain adverse effects that can be controlled<sup>64</sup>. This strategy also applies to  
339 the emergence of new pathogens for which no treatment is yet available. During the SARS-  
340 CoV-2 outbreak, our lab proposed in that way a combination of an antimalarial and an  
341 antibiotic, hydroxychloroquine and azithromycin, based on the results of *in vitro* tests<sup>71</sup>.

342

343

344 **Conclusion**

345 In this study, we investigate the *in vitro* efficacy of 1,280 FDA-approved drugs among 8  
346 MDR and XDR clinical bacteria displaying various resistance phenotypes and resistance  
347 genes. We screened a library of 1,280 drugs against a collection of bacteria. A total of 107  
348 compounds from 9 different therapeutic classes inhibited >90% growth of our GN and GP  
349 bacteria. In view of the hits found, we noticed that the difficulty of finding active compounds  
350 on GN bacteria is greater than for GP bacteria. This reflects our clinical practice issues with  
351 the MDR GN bacteria, and especially the non-fermenting GN.  
352 Some of these results represent a first step towards the use of a drug repurposing strategy for  
353 the treatment of MDR bacterial infections. Those molecules must be further studied on a case-  
354 by-case basis to verify their antibacterial efficacy, understand their mechanisms of action and  
355 confirm their suitability for a relevant administration. This protocol is, after all, becoming  
356 established in our laboratory as routine clinical screening for patients with XDR bacterial  
357 infection.

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366 The authors declare that they have no competing interests.

367 **Authors' contributions:**

368 **Peyclit L., Baron S.A., and Rolain J.-M** designed the study, drafted and revised the  
369 manuscript. **Peyclit L.** performed microbiology analyses. All authors have read and approved  
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## Tables and Figures

**Table 1.** Antimicrobial susceptibility profiles and characterization from our panel according to the study of A.-P. Magiorakos *et al*<sup>3</sup>. MDR: multidrug-resistant; XDR: extensively drug-resistant

**Table 2.** Hits that are not from “infectiology” therapeutic class but from all other classes.

**Table 3.** Possible alternative solutions suitable for our XDR strains

**Figure 1.** Hits were founded after an *in vitro* screening of the 1,280 drugs library for each bacterium according to their therapeutic class. On the left, we discarded the duplicates and we found 107 hits for all the GP and GN. Some drugs are classified in various therapeutic classes and can be either antibacterial, antifungal, or antiviral, so we consider first the antibacterial effect in our counting or the major known effect. MDR: multidrug-resistant; XDR: extensively drug-resistant, GN: Gram-negative, GP: Gram-positive

**Figure 2.** Common compounds between all GP and GN bacteria. A. Name of the hits between all our stains. B. Number of hits in common between GN or GP bacteria.

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 625 resistant

Bacteria	N°	Characterization <sup>3</sup>	Non-susceptibility to at least one agent in all those classes	Known resistance genes
<i>S. aureus</i>	P1943 <sup>72</sup>	MDR	Fluoroquinolones, Anti-staphylococcal $\beta$ -lactams, Glycopeptides, Macrolides	<i>mecA</i> , <i>gyrA</i> , <i>aaD</i> , <i>bleO</i> , <i>ermC</i> , <i>ABC transporters</i>
<i>E. faecium</i>	P5015	MDR	Aminoglycosides, Glycopeptides, Tetracyclines	<i>van A</i>
<i>E. coli</i>	DSM 105182 <sup>73</sup>	MDR	Polymyxins, Tetracyclins, Fluoroquinolones	<i>mcr-1</i>
<i>K. pneumoniae</i>	P9495	MDR	Aminoglycosides, Penicillins + $\beta$ -lactamase inhibitors, Carbapenems, Extended-spectrum cephalosporins, Fluoroquinolones, Folate pathway inhibitors, Monobactams, Polymyxins	<i>OXA-48</i>
<i>A. baumannii</i>	P1887 <sup>74</sup>	MDR	Aminoglycosides, Carbapenems, Fluoroquinolones, Penicillins + $\beta$ -lactamase inhibitors, Extended-spectrum cephalosporins, Folate pathway inhibitors	<i>OXA-51</i> , <i>OXA-23</i> , <i>NDM-1</i>
<i>P. aeruginosa</i>	P6540	XDR	Aminoglycosides, Antipseudomonal cephalosporins, Antipseudomonal fluoroquinolones, Penicillins + $\beta$ -lactamase inhibitors, Monobactams, Polymyxins	
<i>B. multivorans</i>	P6539	XDR	Aminoglycosides, Carbapenems, Cephalosporins, Fluoroquinolones, Penicillins + $\beta$ -lactamase inhibitors, Monobactams, Folate pathways inhibitors, Glycylcyclines, Polymyxins	
<i>P. nosoerga</i>	P8103	XDR	Aminoglycosides, Carbapenems, Cephalosporins, Fluoroquinolones, Penicillins + $\beta$ -lactamase inhibitors, Rifamycins, Folate pathways inhibitors, Tetracyclins, Phosphonic acids, Polymyxins	<i>OXA-158</i>

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630 **Table 2.** Hits that are not in “infectiology” therapeutic class but in all other classes.

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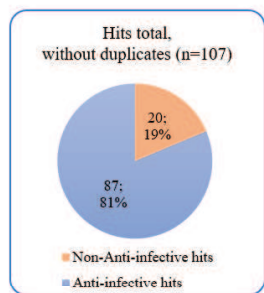
	Hits except "infectiology" class
<i>S. aureus</i> P1943	Dronedarone hydrochloride (Cardiovascular) Thonzonium bromide (Dermatology) Auranofin (Metabolism) Pinaverium bromide (Neuromuscular) 5-fluorouracil, Carmofur, Gemcitabine (Oncology)
<i>E. faecium</i> P5015	Amiodarone, Dronedarone hydrochloride (Cardiovascular) Clomiphene citrate (Z, E) (Endocrinology) Auranofin (Metabolism) Tamoxifen citrate, Gemcitabine, Carmofur, Floxuridine, Pemetrexed disodium, Raltitrexed, 5-fluorouracil, Methotrexate (Oncology)
<i>E. coli</i> DSM 105182	Azaperone, Lomerizine hydrochloride (Central Nervous System)
<i>P. nosoerga</i> P8103	Deferoxamine mesylate (Hematology)

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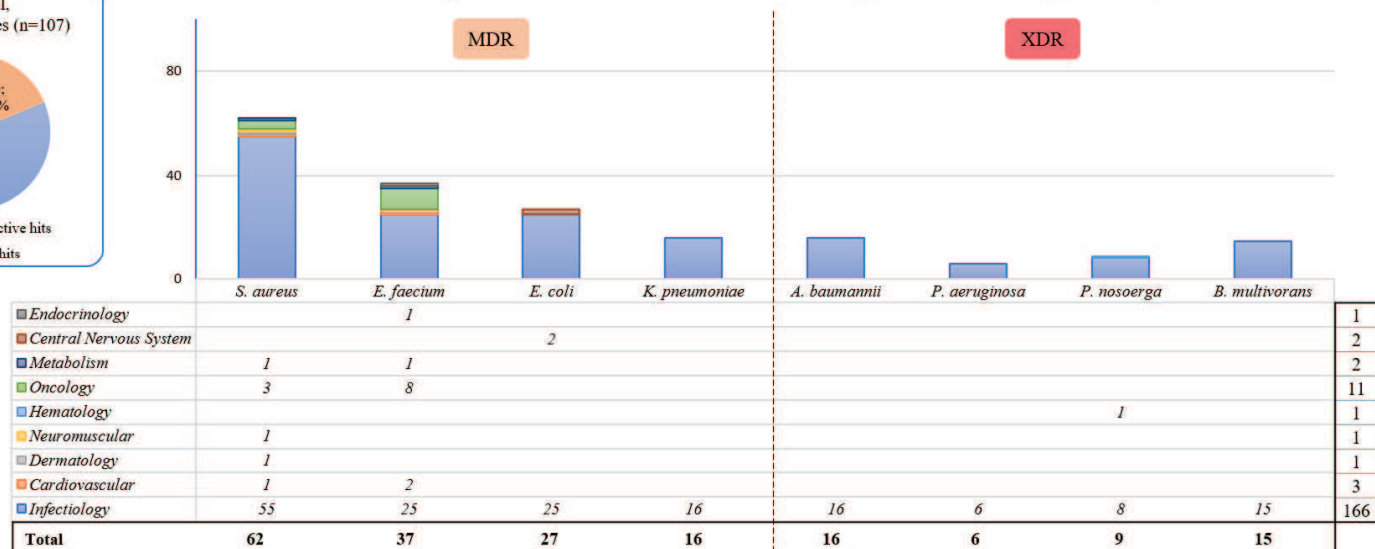
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634 **Table 3.** Possible alternative solutions suitable for our XDR strains

	Combination therapy	Drug repurposing	Increasing dosage regiments
<i>Pseudomonas aeruginosa</i>	Rifampicin + Imipenem Colistin + Imipenem	Auranofin (+ colistin +/- ceftazidime)	Colistin
<i>Pandoraea nosoerga</i>	Rifampicin + Minocyclin	Deferoxamine + ascorbic acid (+ gentamicin)	Rifampicin
<i>Burkholderia multivorans</i>	Fluoroquinolones + $\beta$ -lactams	-	Fluoroquinolones



### Therapeutic class of hits founded among the 1,280 drugs library



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638 **Figure 1.** Hits were found after an *in vitro* screening of the 1,280 drugs library for each

639 bacterium according to their therapeutic class. On the left, we discarded the duplicates and we

640 found 107 hits for all the GP and GN. Some drugs are classified in various therapeutic classes

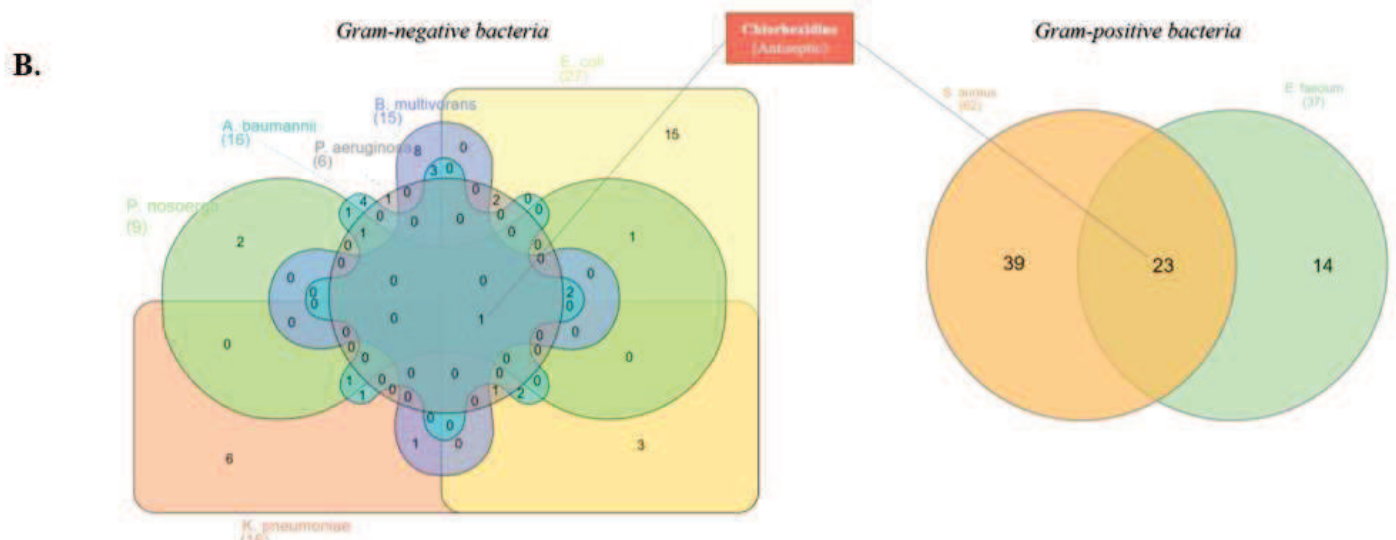
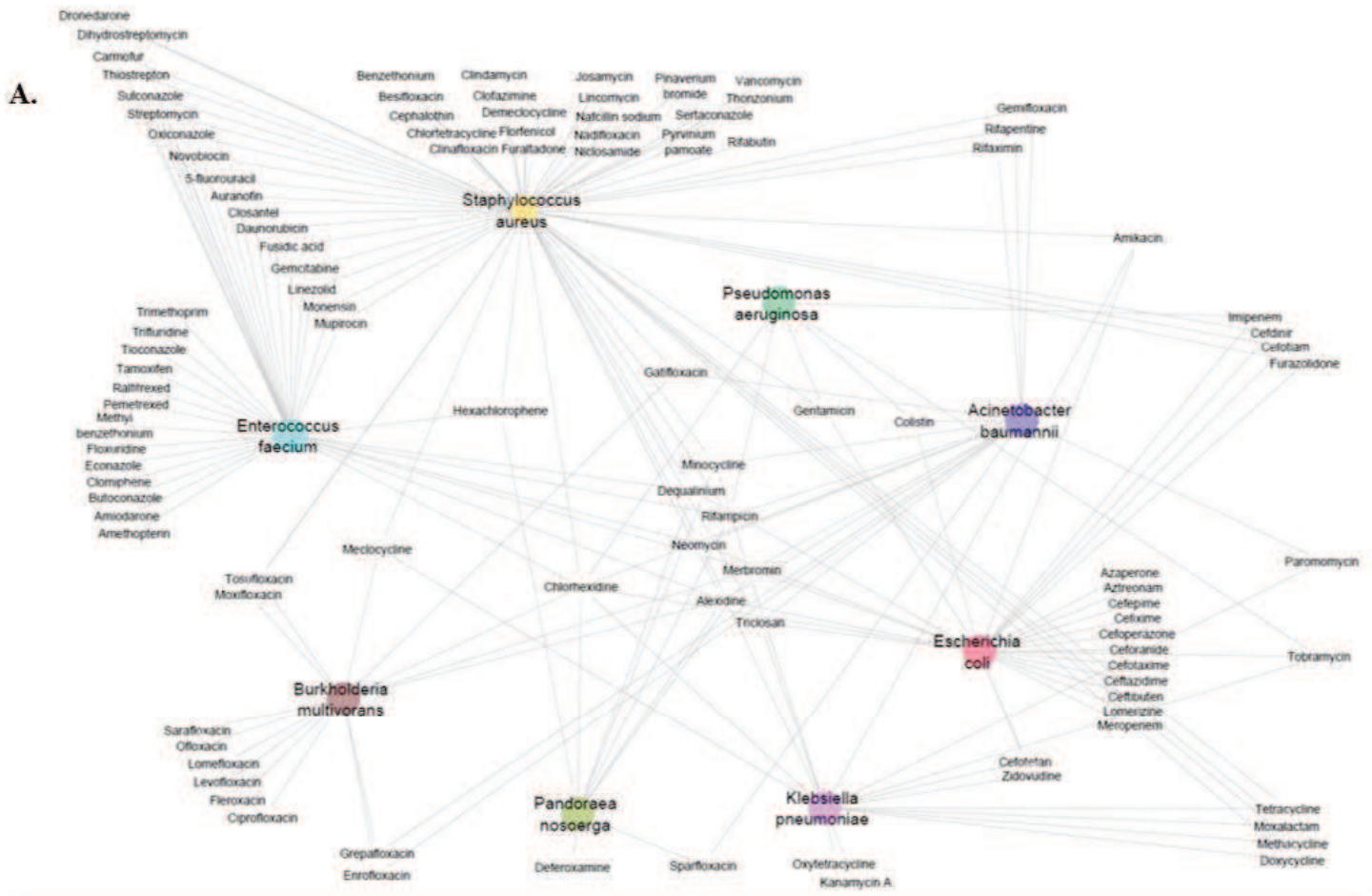
641 and can be either antibacterial, antifungal, or antiviral, so we consider first the antibacterial

642 effect in our counting or the major known effect. MDR: multidrug-resistant; XDR:

643 extensively drug-resistant, GN: Gram-negative, GP: Gram-positive

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647 **Figure 2.** Common compounds between all GP and GN bacteria. A. Name of the hits between  
 648 all our stains. B. Number of hits in common between GN or GP bacteria.  
 649  
 650 **Annex 1.** Comparison of non-anti-infective hits with human PK data from literature.

		Pemetrexed disodium	470,4	4,7	Go further	Cmax: <b>119 µg/mL (119 mg/l)</b> for 500 mg/m <sup>2</sup> by infusion	(Kavathiya et al. 2017)
		Raltitrexed	458,0	4,6		Cmax: <b>652.9 ng/ml (0.6 mg/l)</b> with single intravenous dose of 3 mg/m <sup>2</sup>	(Judson et al. 1998)
		5-fluorouracil	130,0	1,3		Cmax: between <b>393.8 - 487 ng/ml (440.4 µg/l)</b> for a dose of 50 mg/kg	(Mattos et al. 2016)
		Amethopterin (Methotrexate)	454,4	4,5		Cmax: <b>0.01 - 0.1 µmol/l (4.5 - 45.4 µg/l)</b> with oral doses of 10-15 µg	(Inoue and Yuasa 2014)
<i>E. coli</i> DSM 105182	Central Nervous System	Azaperone	327,4	3,3		<i>veterinary drug, no PK data on humans</i>	
		Lomerizine hydrochloride	468,5	4,7		Cmax: <b>9.06 µg/l</b> after a dose of 10mg	(Ren et al. 2014)
<i>P. neoerga</i> PS103	Hematology	Deferoxamine mesylate	560,7	5,6		Cmax: <b>80 - 130 pmol/L (44.8 - 728.9 ng/l)</b> with IV bolus of 10 mg/kg	(Porter 2001)

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	Therapeutic class	Hits except "infectiology" class	PM g/mol	Tested C <sup>o</sup> in mg/l (=10 µmol/l)		Data from in vivo studies	References
<i>S. aureus</i> P1943	Cardiovascular	Dronedaron hydrochloride	593,2	5,9		Steady-state plasma concentrations of <b>84 to 167 ng/mL (84/167 µg/l)</b> , with 400 mg twice daily	(Maria and Ioannis 2010)
	Dermatology	Thonzonium bromide	591,7	5,9		<i>external use in an antibiotic suspension, any data about serum concentration</i>	
	Metabolism	Auranofin	679,5	6,8		Cmax: <b>0.025 µg/ml (0.025 mg/l)</b> , after 6mg per day	(Torres et al. 2016)
	Neuromuscular	Pinaverium bromide	591,4	5,9		Cmax: <b>3,609.98 pg/mL (3.6 µg/l)</b> for 100 mg, with a bioavailability very low	(Patiño-Rodríguez et al. 2015)
	Oncology	5-fluorouracil	130,0	1,3		Cmax: between <b>393.8 - 487 ng/ml (440.4 µg/l)</b> for a dose of 50 mg/kg	(Mattos et al. 2016)
Carmofur		257,3	2,6		Serum concentration: lower than <b>0.02 µg/ml (0.02 mg/l)</b> , with daily administrations of 600 mg for more than 28 days	(Sawada, Matsui, and Okudaira 1983)	
Gemcitabine		263,2	2,6	Go further	Cmax: <b>40.9 µg/ml (40.9 mg/l)</b> with administration of 2000mg/m <sup>2</sup> or <b>7.71 µmol/ml (2 mg/l)</b> with infusion at 600 mg/m <sup>2</sup>	(Alharbi et al. 2019; Fogli et al. 2001)	
<i>E. faecium</i> P5015	Cardiovascular	Amiodarone	645,3	6,5	Go further	Cmax: <b>1.17 mg/ml (1.17 g/l)</b> , after oral dose of 600 mg	(Gaete et al. 1995)
		Dronedaron hydrochloride	593,2	5,9		Steady-state plasma concentrations of <b>84 to 167 ng/mL (84/167 µg/l)</b> , with 400 mg twice daily	(Maria and Ioannis 2010)
	Endocrinology	Clomiphene citrate (Z,E)	405,96	4,1		Cmax: <b>15 ng/mL (15 µg/l)</b> after single oral dose of 50 mg	(Ghobadi et al. 2009)
	Metabolism	Auranofin	680,5	6,8		Cmax: <b>0.025 µg/ml (0.025 mg/l)</b> , after 6mg per day	(Torres et al. 2016)
	Oncology	Tamoxifen citrate	371,5	3,7		Cmax: <b>368 nmol/l (136.7 µg/l)</b> for a dose of 20 mg	(Binkhorst et al. 2015)
		Gemcitabine	263,2	2,6	Go further	Cmax: <b>40.9 µg/ml (40.9 mg/l)</b> with administration of 2000mg/m <sup>2</sup> or <b>7.71 µmol/ml (2 mg/l)</b> with infusion at 600 mg/m <sup>2</sup>	(Alharbi et al. 2019; Fogli et al. 2001)
		Carmofur	257,3	2,6		Serum concentration: lower than <b>0.02 µg/ml (0.02 mg/l)</b> , with daily administrations of 600 mg for more than 28 days	(Sawada, Matsui, and Okudaira 1983)
Floxuridine		249,2	2,5		Cmax: <b>4.90 ng/ml (4.90 µg/l)</b> , 400 mg three times daily. If haemodialysis, less.	(Alharbi et al. 2019)	

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## **2. Article 3. *Pandoraea nosoerga*, drug repurposing and whole genome sequencing in combined liver-lung transplantation for cystic fibrosis - Peyclit L., Baron S.A., Reynaud-Gaubert M., Cassir N., et Rolain J-M.**

Soumis dans Journal of Cystic Fibrosis en Août 2019.

A travers ce *case report*, nous avons étudié une situation clinique à laquelle nos cliniciens ont été confrontés à l'hôpital de Marseille, une infection à bactérie « difficile à traiter » et dans lequel nous avons pu appliquer la stratégie du repositionnement moléculaire.

La patiente atteinte de mucoviscidose était colonisée de manière récurrente par *P. nosoerga* au cours des dernières années. Les bactéries opportunistes du genre *Pandoraea* sont fréquemment rencontrées de manière chronique dans les voies respiratoires des patients atteints de mucoviscidose<sup>40,41</sup>. La patiente a reçu en 2018 une transplantation bi-pulmonaire et hépatique pour améliorer son état clinique, accompagnée d'une prophylaxie antibiotique afin de limiter une nouvelle infection bactérienne pouvant fortement nuire à l'efficacité de ses transplantations<sup>42</sup>. Malgré cela, elle a développé à nouveau en post-opératoire une infection à *P. nosoerga* XDR. Les traitements antibiotiques n'étant plus efficaces, nous avons essayé l'alternative thérapeutique du repositionnement moléculaire. Nous avons aussi voulu comprendre l'origine de son infection donc nous avons étudié par comparaison génomique ses souches pré- et post-greffes et celle de son frère ancien porteur de *P. nosoerga*.

1 **Title:** *Pandoraea nosoerga*, drug repurposing and whole genome sequencing in a combined  
2 liver-lung transplantation for cystic fibrosis

3 **Running title:** *Pandoraea nosoerga* and lung transplantation

4 **Keywords:** cystic fibrosis, *Pandoraea*, Drug repurposing, comparative genomic, extensively  
5 drug-resistant bacteria

6  
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19 Summary: 99

20 Text: 1197/1200

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22 Figures/tables: 1

23 Annex: 1

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25

26 **Abstract**

27 A 26-year-old girl with a longstanding colonization by *Pandoraea nosoerga*, underwent liver-  
28 lung transplantation for cystic fibrosis (CF) in 2018. Her brother also suffering from CF was  
29 also colonized by *P. nosoerga*. Despite appropriate perioperative antibiotic therapy, she had  
30 post-transplant bacteremic pneumonia caused by extensively drug-resistant *P. nosoerga*. Drug  
31 repurposing was used to optimize treatment options. The source of colonization was studied  
32 by comparative whole genome sequencing including pre- and post-transplant strains and her  
33 brother's strains. The source of infection appeared to be her own pre-transplant strain,  
34 emphasizing the urgent need to study and implement effective decontamination protocols  
35 before transplantation.

36

37

38

39



## 40 Introduction

41 *Pseudomonas aeruginosa*, *Burkholderia spp.* or *Pandoraea spp.* are opportunistic bacteria  
42 that chronically colonize the respiratory tract of cystic fibrosis (CF) patients[1]. Lung  
43 transplantation provides a way to overcome respiratory failure at end-stage CF[2]. However,  
44 this process involves high risks of infection, particularly by these persistent bacteria, which  
45 are associated with poor post-transplant outcomes[3,4]. Chaparro C. *et al.* found that CF  
46 patients infected with *B. cepacia* had significantly earlier mortality than those who were not  
47 infected[5]. Antibacterial prophylaxis after lung transplantation is recommended using  
48 ceftazidime as first choice[6], but no clear preventive treatment guidelines are given for CF  
49 patients[7]. Moreover, treating post-transplant infections caused by these bacteria with  
50 various resistant determinants[8] may be challenging.

51 In this case report, the patient with a longstanding colonization by *Pandoraea nosoerga*,  
52 underwent liver-lung transplantation for CF[9]. Despite appropriate perioperative antibiotic  
53 therapy, she had post-transplant bacteremic pneumonia caused by extremely drug-resistant  
54 (XDR) *P. nosoerga*. Drug repurposing was used to optimize treatment options and whole  
55 genome sequencing (WGS) to analyze the source of this post-transplant infection.

56

## 57 Case presentation

58 The patient was a 21-year-old girl who presented at 7 years-old a steatorrhea secondary to  
59 pancreatic exocrine insufficiency. Genetic testing revealed homozygous Phe508del mutation  
60 in the CF transmembrane conductance regulator gene confirming the diagnosis of CF.

61 Chronic recurrent lung infections ensued from childhood with multiple bacteria including  
62 *Staphylococcus aureus* sensitive to methicillin, *P. aeruginosa*, and Extensively Drug Resistant  
63 (XDR) *Pandoraea nosoerga* (**Table S1**). Her history included CF-associated liver disease  
64 leading progressively to cirrhosis. In the two years prior to transplantation, there were a

65 significant decrease in lung function (maintenance oxygen therapy: nasal cannula at 4-6 l/min,  
66 non-invasive ventilation), hospitalizations for recurrent infections and complications of liver  
67 disease. Combined liver-lung transplantation was performed under extracorporeal membrane  
68 oxygenation (ECMO) in July 2018. Perioperative antibiotic prophylaxis consisted of  
69 piperacillin/tazobactam and tigecycline. The immunosuppressive regimen consisted of IV  
70 cyclosporin and corticosteroids. She did not receive immunosuppressive induction neither  
71 mycophenolate mofetil therapy. Prophylaxis with ganciclovir was also prescribed according  
72 to her CMV status (D+/R+). On Day 5 post-transplant, a left-lung ischemia conducted to a  
73 surgical explanation and was complicated by a first septic shock due to *P. nosoerga* isolated  
74 from perioperative samples, blood cultures and broncho-alveolar lavage. Bacterial  
75 identification was performed using MALDI-TOF, which initially identified the strains as *P.*  
76 *pulmonicola* but DNA-DNA hybridization reclassified all the strains as *P. nosoerga*.  
77 Susceptibility assays were done using the disk diffusion method on a Mueller-Hinton agar  
78 (BioMérieux, Marcy l'Etoile, France) (**Table S1**). As no breakpoints for *Pandora* spp. are  
79 available in the European Committee of Antimicrobial Susceptibility Testing guidelines, we  
80 used recommendations for *Burkholderiaceae* spp. The antibiotic therapy was switched to  
81 intravenous meropenem plus tigecycline and minocycline administered *via* the nasogastric  
82 tube with an initial good response. On Day 14 post-transplant, she had a second septic shock  
83 cause by *P. nosoerga* isolated from blood cultures without signs of infective endocarditis on  
84 transesophageal echocardiography nor septic thrombophlebitis on tomodesitometry scan. A  
85 1,280 panel of non-anti-infective and anti-infective drugs (Prestwick, Illklich, France) was  
86 tested (*unpublished data*) and led to switch again with doxycycline plus rifampicin and  
87 risperidone. Daily chlorhexidine gluconate 2% bathing was also implemented. On Day 21,  
88 recurrent bacteremia due to *P. nosoerga* conducted to the replacement of ECMO cannulation.

89 Despite this, the patient died on Day 25 because of refractory septic shock caused by XDR *P.*  
90 *nosoerga* (**Figure 1A**).

91

## 92 Genomic methods

93 Four *P. nosoerga* strains were isolated from two sputum expectorations (P9489 in 2012,  
94 P9490 in 2016), one blood sample from our patient after her liver-lung transplantation in 2018  
95 (P8103), and one sputum expectoration (P9491) from her brother with a longstanding  
96 colonization by *P. nosoerga* since 2012 (**Figure 1A**). WGS was performed on these strains  
97 with the MiSeq sequencer (Illumina Inc., San Diego, USA) using a paired-end strategy and  
98 assembled using Spades software[10]. Genomes were submitted under GenBank accession  
99 number JABVYB000000000 for P8103, JABVYA000000000 for P9489,  
100 JABVXY000000000 for P9490 and JABVXZ000000000 for P9491. We looked for  
101 antimicrobial resistance genes with ABRicate using Resfinder and ARG-ANNOT  
102 databases[10]. Pangenome was performed using Roary tool with default parameters[10]. The  
103 Roary core genome alignment was then used to infer a maximum-likelihood phylogenetic tree  
104 of the isolates and *P. nosoerga* LMG31109[11] as reference using RAxML software. All our  
105 strains were qualified as extensively drug-resistant (XDR)[12] and carried a *bla*<sub>OXA-158</sub> gene  
106 with an identity of 99.53% (**Table S1**). They had a G+C content of 66.1% and genome  
107 lengths were around 5 Mbp (4.9 Mbp for P8103, P9490 and P9491 and 5.1 Mbp for P9489).  
108 Pangenome was composed of 4,420 genes including 4,161 core genes and 259 shell genes  
109 (**Figure 1B**). Average Nucleotide Identity (ANI) scores, calculated with JSpeciesWS tool and  
110 using the P8103 genome as reference strain, were 99.68% for P9489 and 99.69% for P9490  
111 and P9491. We analyzed single nucleotide polymorphisms (SNP) on the core genome with  
112 Snippy[10]. The reference genome P8103 had only 58 SNPs differences on the core genome  
113 with the strain from 2016 (P9489), whereas there were 86 and 113 SNPs with the first isolated

114 strain in 2012 (P9490) and the brother's one (P9491) respectively. Only 27 SNPs were  
115 recovered between the 2012 strains from the brother and the sister. These results showed that  
116 strains are closely related with a mutation rate estimated between 2.9 and  $6.10^{-6}$ /site/year. The  
117 strains isolated from 2012 in the sister (P9490) and brother (P9491) were the closest,  
118 suggesting they were colonised by the same clone (**Figure 1B**).

119

## 120 **Conclusion**

121 *Pandora* spp. infections are commonly described in CF patients[1,13,14]. Yet, this genus  
122 does not remain absolutely known and we have confirmed that misidentifications are  
123 possible[13,14]. In 2019, Peeters C *et al.* described 17 new species of *Pandora* including *P.*  
124 *nosoerga*[11]. Although *Pandora* spp. are originally environmental bacteria, *P. nosoerga*  
125 was found in samples from CF patients around the world[11]. Risk of fatal outcome following  
126 a pulmonary transplantation in CF patient chronically colonized by *Pandora* is high in  
127 literature[4,14], and this case report highlights the difficulty to prevent the infection directly  
128 after transplantation.

129 The high-throughput screening broadens treatment solutions when conventional solutions fail  
130 with an easy and rapid way[12]. Indeed, drug repurposing in the treatment of “difficult to  
131 treat” bacteria is a promising alternative to overcome resistance patterns, allowing the use of  
132 molecules with mechanisms and targets different from standard antibiotics[12]. However,  
133 most of the hits were antiseptics that are not useful for systemic use so antibiotic  
134 combinations or increased doses regimens when it is possible were rather preferable  
135 (*unpublished data*).

136 Our results showed that the patient appears to have recolonized from her own strain,  
137 suggesting insufficient decontamination prior to transplantation and raising the question of  
138 sinuses as a reservoir[15]. Indeed, bath of chlorhexidine could also be used in the pre-

139 transplant phase, in order to eliminate local reservoirs. A CF patient with XDR pathogens was  
140 successfully transplanted after these airways were decontaminated with chlorhexidine,  
141 bacitracin and 10% polyvidone-iodine solutions[15]. Decontamination protocols therefore  
142 seems essential for the transplant success in these patients, because cross infections are also  
143 possible as observed between these two siblings or in a Danish study conducted by Jørgensen  
144 I.M. *et al.*[1].

145 Further works are needed to understand the source of the recolonization in order to improve  
146 the decontamination process, to consider possible transmission between siblings and to  
147 prevent this fatal outcome.

148

149

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156

157 **Transparency declaration:**

158 The authors declare that they have no competing interests.

159 **Authors’ contributions:**

160 **Peyclit L., Cassir N., Baron S.A. and Rolain J.-M** designed the study, drafted and revised  
161 the manuscript. **Peyclit L. and Baron S.A.** performed microbiology and genomic analyses.

162 All authors have read and approved the final manuscript.

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211



212

## Tables and Figures

213

214 **Figure: 1**

215 **Figure 1.** A. Timeline of clinical management of the patient. B. Phylogenetic tree based on  
216 the pangenome of the four strains and one reference of *Pandoraea nosoerga*.

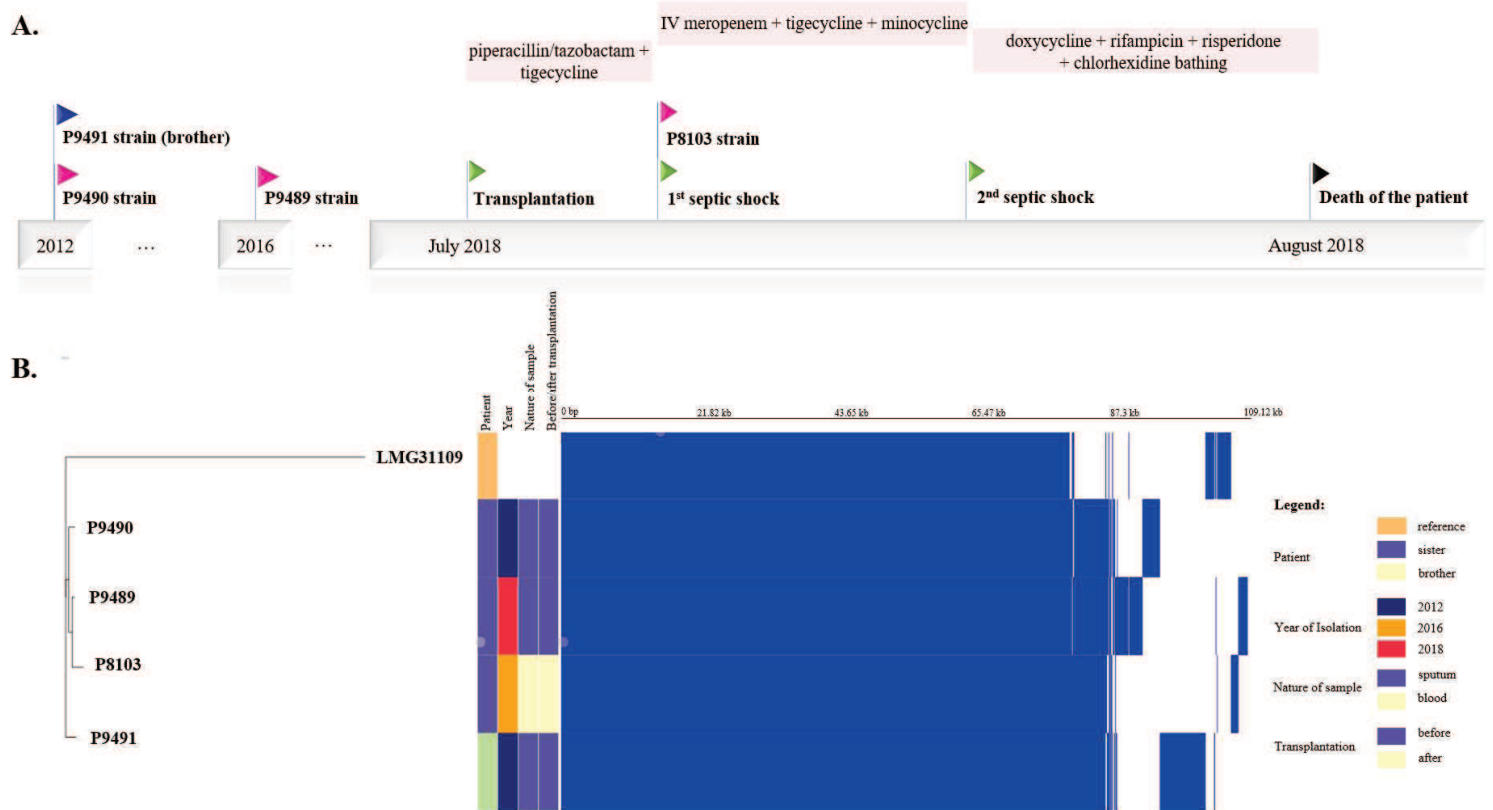
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218 **Supplementary File: 1**

219 **Appendix Table. Table S1.** Bacterial identification and antibiotic susceptibility of our  
220 strains. NT: non tested

221

222



224 **Figure 1.** A. Timeline of clinical management of the patient. B. Phylogenetic tree based on  
 225 the pangenome of the four strains and one reference of *Pandoraea nosoerga*.

226 **Supplementary data**227 **Table S1.** Bacterial identification and antibiotic susceptibility of our strains. NT: non tested.

Strain number		<b>P8103</b>	<b>P9489</b>	<b>P9491</b>	<b>P9490</b>
Year of sampling		2018	2016	2012	2012
MALDI-TOF Identification		<i>Pandoraea pulmonicola</i>	<i>P. pulmonicola</i>	<i>P. pulmonicola</i>	<i>P. pulmonicola</i>
DNA-DNA-hybridization	Identification	<b><i>Pandoraea nosoerga</i></b>	<b><i>P. nosoerga</i></b>	<b><i>P. nosoerga</i></b>	<b><i>P. nosoerga</i></b>
	Score (%)	99.2	99.2	99.2	99.2
	Reference	<i>Pandoraea nosoerga</i> (LMG 31109)			
Antimicrobial resistance	Resistance gene	<i>bla<sub>OXA-158</sub></i>	<i>bla<sub>OXA-158</sub></i>	<i>bla<sub>OXA-158</sub></i>	<i>bla<sub>OXA-158</sub></i>
	Identity (%)	99.53	99.53	99.53	99.53
Polymyxins	Colistin	R	R	R	R
Penicillins	Ticarcillin	R	R	R	R
Penicillins + $\beta$ -lactamase inhibitors	Ticarcilin/Clavulanate	R	S	R	R
	Piperacillin/Tazobactam	R	R	R	R
Cephalosporines	Ceftazidime	R	R	R	R
	Cefepime	R	R	NT	NT
Monobactams	Aztreonam	R	R	NT	NT
Carbapenems	Imipenem	R	R	R	R
	Meropenem	R	R	NT	NT
Aminoglycosides	Gentamicin	R	NT	R	R
	Tobramycin	R	R	R	R
	Amikacin	R	R	R	R
Folate pathway inhibitors	Trimethoprim/Sulfamethoxazole	R	S	R	R
Fluoroquinolones	Ciprofloxacin	R	R	R	R
Rifamycines	Rifampicin	R	R	I	NT
Phosphonic acids	Fosfomicin	R	R	R	R
Tetracyclines	Doxycyclin	S	S	NT	NT

228

### 3. Conclusion

L'étude et la mise en pratique du repositionnement moléculaire sont prometteurs pour le traitement des BMR. Les cliniciens peuvent choisir de réaliser cette méthode s'ils se rendent compte que leurs moyens de traitement sont limités dans des infections à des bactéries difficiles à traiter. Cette stratégie est économique et facile à réaliser au laboratoire ; l'administration des médicaments efficaces peut être rapide et sûre dans la mesure où la bibliothèque ne comprend que des molécules déjà approuvées par les agences de santé. Ceci constitue une réponse à une médecine personnalisée, en fonction des résistances de la bactérie et des données du patient.

Cependant, certains médicaments efficaces dans notre criblage pourraient être rejetés par méfiance des cliniciens : ces molécules présentent un risque de toxicité non négligeable et nécessitent une surveillance rapprochée, comme l'amiodarone à marge thérapeutique étroite ou des anti-cancéreux. Par ailleurs, nous avons remarqué que seules quelques molécules pouvaient être efficaces sur certaines de nos BMR, bien qu'ayant criblé 1280 médicaments. Dans ce cas, l'utilisation d'associations médicamenteuses doit être une thérapeutique envisagée, qui est évaluée *in vitro* par *checkerboard* au laboratoire pour détecter d'éventuels effets antagonistes. L'augmentation des concentrations d'antibiotiques peut aussi être considérée pour lutter contre ces BMR. Dans certains cas, la résistance a été surmontée grâce à la concentration fixe arbitrairement choisie pour le criblage, supérieure à celle utilisée habituellement en clinique : les bactéries devenaient sensibles. Enfin, le choix d'utiliser une molécule doit toujours être confirmé avant son utilisation par la détermination de sa concentration minimale inhibitrice avec des tests de sensibilité comme les CMI.

Lors de ces travaux, nous avons démontré que l'appartenance à une classe thérapeutique n'est pas fixée à une maladie ou un pathogène. En effet, les organismes procaryotes et eucaryotes partagent des processus physiologiques universels, qui sont ciblés par des molécules thérapeutiques. En effet, plusieurs hits n'appartenant pas à la classe des antibiotiques se sont avérés efficaces contre la croissance de ces micro-organismes. La zidovudine est un antirétroviral découvert en 1985 qui a inhibé à 100% la croissance des entérobactéries de notre panel. Par sa faible toxicité et les concentrations sériques retrouvées chez l'homme, elle constitue à nos yeux une piste encourageante dans le repositionnement moléculaire contre certaines de ces BMR.

## V. Zidovudine, une molécule efficace contre les bactéries résistantes à la colistine et aux carbapénèmes

La classe des analogues nucléosidiques, pharmacologiquement diversifiée et issue de ribose naturel chimiquement modifié, est largement utilisée comme agents anticancéreux et antiviraux. Les analogues de nucléosides imitent les nucléosides endogènes, en exploitant le métabolisme cellulaire et en s'incorporant à la fois dans l'ADN et l'ARN<sup>43</sup>. Cette propriété rend ces molécules efficaces pour inhiber la réplication virale et arrêter la prolifération des cellules cancéreuses. Ces dernières années, leurs propriétés antibactériennes ont été de plus en plus rapportées<sup>43,44</sup>. Nos précédents travaux sur la recherche de molécules présentes dans nos pharmacies contre les bactéries multi-résistantes nous ont ainsi mené à considérer un de ses analogues, la zidovudine (ou azidothymidine, AZT).

La zidovudine ou chimiquement nommée 3'azido-3'-deoxythymidine est un analogue de la thymidine avec un groupe azido à la place du groupe hydroxyle sur le carbone 3' du cycle désoxyribose. Elle agit sur la synthèse de l'ADN comme « terminateur de chaîne » : le groupe azido va empêcher la formation de la liaison phosphodiester 5' à 3' essentielle à l'allongement de la chaîne d'ADN. Les enzymes intracellulaires thymidine kinase, thymidylate kinase et nucléoside diphosphate kinase respectivement permettent son activation grâce à une tri-phosphorylation.

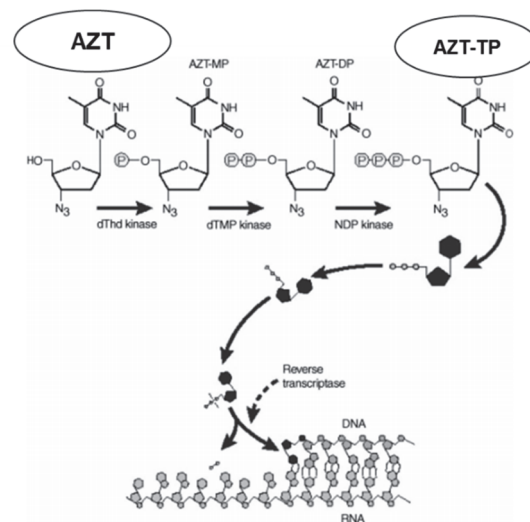


Figure 2. Mécanisme d'action de la zidovudine

Elle a une plus grande affinité pour la transcriptase inverse virale du VIH que pour l'ADN polymérase humaine ; elle a été le premier antirétroviral commercialisé pour traiter les patients infectés du VIH, sous le nom de Rétrovir®, et est disponible en comprimés, sirop ou par voie IV. Les effets indésirables communs sont des troubles digestifs, des maux de tête ou la perte d'appétit. Plus rarement, des effets

toxiques majeurs peuvent se présenter, d'ordre hématologique avec des anémies, des neutropénies ou d'ordre métabolique avec des acidoses lactiques, des hépatites ou des cardiomyopathies. Son activité antibactérienne a été découverte en 1987 contre diverses entérobactéries<sup>45</sup>. En revanche, l'AZT semble être inefficace contre les cocci à GP telles que les Staphylocoques et *E. faecalis*, ainsi que contre les espèces de Mycobactéries et *P. aeruginosa*<sup>45-47</sup>.

Suite au criblage et aux données de la littérature, nous avons choisi de tester l'efficacité de l'AZT in vitro sur un panel d'entérobactéries (*E. coli* et *K. pneumoniae*) résistantes aux carbapénèmes et à la colistine dans l'**Article 4** « *Zidovudine: a salvage therapy for mcr-1 plasmid-mediated colistin-resistant bacterial infections?* ». Nos résultats et leur comparaison aux concentrations utilisées chez l'homme nous permettaient de repositionner cette molécule en tant que piste prometteuse pour le traitement de ces BMR.

En 1990, Lewin C. S. et co-auteurs rapportaient des souches résistantes à l'AZT dans des échantillons fécaux de patients atteints du SIDA et traités par AZT<sup>48</sup>. Aucune donnée n'était disponible quant à la prévalence de sa résistance chez les bactéries. Celle-ci semblait être expliquée par la perte de l'activité de la thymidine kinase, requise pour l'activation de l'AZT ou par un autre mécanisme non élucidé<sup>47</sup>. Doléans-Jordheim et coauteurs ont isolé in vitro après exposition à l'AZT des clones résistants et étudié la thymidine kinase de ces souches<sup>49</sup>. L'**Article 5** « *Inactivation of thymidine kinase as a cause of resistance to zidovudine in clinical isolates of Escherichia coli: a phenotypic and genomic study* » a ainsi permis d'établir cette prévalence et d'étudier davantage la thymidine kinase, l'enzyme cible potentiellement mise en cause dans la résistance de ces souches.

Enfin, ces résultats soulignant que les isolats d'*E. coli* résistants à l'AZT étaient relativement fréquents, nous avons préféré étudier son utilisation en association avec d'autres molécules plutôt qu'en monothérapie pour limiter l'apparition de résistance et favoriser les synergies d'action<sup>50</sup>. Son efficacité en combinaison avec la tigécycline contre des entérobactéries résistantes aux carbapénèmes a été démontré in vitro<sup>51</sup>. Par ailleurs, l'activité in vitro et in vivo de l'AZT en combinaison avec la colistine a été démontré contre 74 entérobactéries, productrices de carbapénémases NDM-1, de BLSE ou porteuses du gène *mcr-1*<sup>52</sup>. Une étude réalisée dans l'équipe du Pr. Jian Li en 2019 a démontré le potentiel synergique de la combinaison de la polymyxine B et de l'AZT contre des souches de *K. pneumoniae* productrices de NDM. C'est donc dans le cadre d'une collaboration avec cette équipe à l'université de Monash (Melbourne, Australie) que nous avons étudié les paramètres PK/PD de la combinaison colistine et AZT contre deux souches cliniques de *K. pneumoniae* sensible ou résistante à la colistine. Ceci a fait l'objet de l'**Article 6** « *Colistin and zidovudine, a synergic combination to fight clinical colistin-resistant K. pneumoniae* ».

# **1. Article 4. *Zidovudine: a salvage therapy for mcr-1 plasmid-mediated colistin-resistant bacterial infections?* - Peyclit L.\*, Baron SA.\*, Yousfi H., et Rolain J-M.**

\* contribution égale

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Grâce au screening de 1280 molécules, nous avons pu mettre en évidence l'efficacité de la zidovudine sur la croissance d'*E. coli* et *K. pneumoniae*. Une autre équipe de recherche a reconnu l'efficacité de l'AZT sur des entérobactéries productrices de carbapénémases par la même approche de screening phénotypique<sup>51</sup>. L'objectif de notre travail était donc d'évaluer l'efficacité de l'AZT sur des souches d'entérobactéries résistantes à la colistine, mais également de confirmer l'efficacité sur des souches résistantes aux carbapénèmes. Les CMI de l'AZT ont été réalisées sur 40 entérobactéries, par la méthode de microdilution, en duplicate et par deux personnes différentes. Nous avons donc montré l'efficacité de cette molécule à la fois sur les souches d'*E. coli* et de *K. pneumoniae* sécrétrices de carbapénémases (OXA-48, NDM, KPC) mais également sur des souches résistantes à la colistine, incluant des souches portant le gène *mcr-1*. La comparaison des CMI avec les concentrations sériques d'AZT suggère que cette molécule représente un véritable intérêt pour le traitement des infections à BMR.

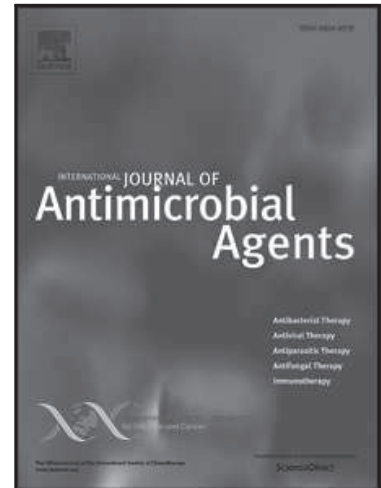


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Zidovudine: a salvage therapy for mcr-1 plasmid-mediated colistin-resistant bacterial infections?

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## Zidovudine: a salvage therapy for *mcr-1* plasmid-mediated colistin-resistant bacterial infections?

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**Keys words:** multi-drug resistant Gram negative bacteria; *mcr-1*; zidovudine; last line antibiotic; azidothymidine

Over the last decade, antibiotic resistance has become a major new threat to human health and a worldwide problem prompting the WHO to prioritize the fight against multidrug-resistant bacteria. The WHO positioned last year *Enterobacteriaceae* as an antibiotic-resistant priority pathogen [1]. Among all threats, the newly described *mcr-1* plasmid-mediated colistin-resistant mechanism [2], which has been described all over the world in Gram-negative bacteria [3], became the most important. We have recently reported that this threat is probably overestimated since many old antibiotics are still effective and could be reintroduced into the pharmaceutical market to treat such infections [4–6]. The main problem is that those old but useful antibiotics are no longer available in many countries [7]. What could solve both problems of antibiotic-resistance and no-longer available drugs in the market is "drug repurposing" i.e the use of a FDA-approved drug as new therapeutic agents. Using this

approach by screening a total of 1,163 FDA-approved drugs, Ng *et al* have recently found that zidovudine, an antiviral drug active against HIV, was effective in vitro against 13 carbapenem-resistant and colistin-susceptible *Enterobacteriaceae* (*Escherichia coli* and *Klebsiella pneumoniae*) with MICs compatible with human use [8]. Zidovudine, a nucleoside reverse transcriptase inhibitor, was the first antiretroviral commercialized for the treatment of HIV infection in 1987 [9]. This nucleotide and synthetic analog was initially used as an anti-cancer drug [9], but, from 1986, its antibacterial effect was revealed in bacteria [10,11] that possess a thymidine kinase homolog able to activate the drug [12]. Using a similar approach with a different FDA-approved library of 1,280 drugs (Prestwick, Illkirch, Graffenstaden, France), we also identified zidovudine as an effective drug against *Enterobacteriaceae*. To our knowledge, no study already reported zidovudine efficacy on colistin-resistant Gram-negative bacteria. In our laboratory, we collected a high number of multi-drug resistant *Enterobacteriaceae* including carbapenem and colistin-resistant strains isolated from different geographical areas worldwide for which molecular mechanisms of resistance have been previously characterized. We report here the efficacy of this compound against a series of 40 strains including 16 *Escherichia coli* (12 colistin-resistant isolates, 3 carbapenem-resistant strains and 1 susceptible isolate) and 22 *Klebsiella pneumoniae* (11 colistin-resistant strains, 9 carbapenem-resistant isolates, one colistin- and carbapenem-resistant strain and one susceptible isolate). Molecular mechanisms of resistance to colistin include *mcr-1*, *mgrB* inactivation and *PmrB* mutation whereas resistance to carbapenems include *bla*<sub>NDM</sub>, *bla*<sub>KPC</sub> and *bla*<sub>OXA-48</sub> carbapenemase; details are given in Table 1. Two strains of *Staphylococcus aureus* which are intrinsically resistant to zidovudine were also used as controls [13] (Table 1).

Zidovudine MICs were determined using broth microdilution dilution method in Mueller Hinton II broth with a zidovudine (Sigma-Aldrich, Missouri, United States) concentration

ranging from 0.2 to 100  $\mu\text{M}$  (i.e. 0.053  $\mu\text{g}/\text{mL}$  to 26.7  $\mu\text{g}/\text{mL}$ ). All strains were susceptible to zidovudine with MICs ranging from 0.2 to 6.25  $\mu\text{M}$  i.e from 0.05  $\mu\text{g}/\text{mL}$  to 1.67  $\mu\text{g}/\text{mL}$  (Table 1). Interestingly, zidovudine was also highly effective against a clinical isolate that was both carbapenem and colistin-resistant (*K. pneumoniae* strain 853; Table 1). The two *S. aureus* strains were resistant with MICs  $>100 \mu\text{M}$  ( $>26.7 \mu\text{g}/\text{mL}$ ).

From a clinical point of view these findings are extremely important for the medical community because of the pharmacokinetic properties of zidovudine. In a study with healthy volunteers, a  $C_{\text{max}}$  of 4  $\mu\text{M}$  (1.07  $\mu\text{g}/\text{ml}$ ) of zidovudine was obtained 1h after the administration of 200 mg of zidovudine in a single oral administration [14]. Similarly, Wattanagoon *et al* have reported in healthy volunteers in Thailand a  $C_{\text{max}}$  of 17.98  $\mu\text{M}$  (4.8  $\mu\text{g}/\text{ml}$ ) after a single dose of 300 mg of zidovudine [15]. Zidovudine is used at the dose of 600 mg/day (300 mg twice a day) in HIV treatment suggesting that a plasmatic concentration above the MIC could be easily achieved. This dosage could be increased for example to 2400 mg/day (600 mg four times/day) since over dosage of 20g has been reported to be free of side effects in the past in humans [16]. Moreover, zidovudine is currently available either in tablet form or in intravenous form that could be used as a slow intravenous infusion (half-life of zidovudine is 1.1 hour) in case of severe sepsis or meningitis since zidovudine can achieve therapeutic concentrations in the cerebrospinal fluid [17]. Zidovudine could also be used during pregnancy and in children [18]. Finally, side effects, as hematotoxicity, are well known and are associated with long-term administration of zidovudine so they could easily be managed as salvage therapy if used for a relatively short period of time.

One question with the use of zidovudine as salvage therapy for multidrug-resistant bacterial infections is the possibility of resistance development. This has been already reported in *E. coli* in HIV patients treated with zidovudine [19] due to the loss of thymidine kinase activity [13,20] preventing zidovudine phosphorylation and activation. Thus it seems reasonable to

combine zidovudine with other drugs to circumvent the selection of zidovudine-resistant strains, for example fosfomycin or minocycline that are known to be effective against such strains [5,6] or tigecycline as recently reported by Ng *et al.* [8].

To conclude, our *in vitro* study demonstrates that drug repurposing is an effective way to (re)discover existing drugs that may be able to solve some of the current problems of worldwide antibiotic-resistance. Indeed, the antibacterial activity of zidovudine was previously described in mice [11] and suspected *in vivo* in humans [21]. The revival of old antibiotics can be useful to fight bacteria resistant to multiple current antibacterial drug, both screening of large libraries of approved drug as well as of the literature for old data on antibacterial activities. Here zidovudine is identified as a leader in this context of worldwide emergence of colistin resistance. Case reports and clinical trials with zidovudine in combination with other drugs to treat patients infected with carbapenem-resistant and/or colistin-resistant bacteria definitely prompt us to add this drug to our therapeutic weapons against multi-drug resistant bacteria. As patents on zidovudine expired in 2005, several generics exist and this drug is less used as an antiretroviral therapeutic, its use as an antibacterial drug could be an affordable alternative.

### ***Declarations***

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### **Competing Interests**

None.

### **Ethical Approval**

Not required

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**Table 1.** MIC of colistin, imipenem and zidovudine against various colistin or carbapenemase-resistant strains.

Species	Strain	Origin	Country	Resistance phenotype	Resistance mechanism	MIC colistin (µg/mL)	MIC imipenem (µg/mL)	MIC AZT (µg/mL)	MIC AZT (µmol/L)	References
<i>Escherichia coli</i>	ATCC 25922	human	United States	none	susceptible	1	0.19	0.835	3.125	ATCC
<i>E. coli</i>	LH1	human	Laos	colistin	<i>mcr-1</i>	6	0.125	0.104	0.4	[20]
<i>E. coli</i>	LH30	human	Laos	colistin	<i>mcr-1</i>	6	0.19	0.208	0.8	[20]
<i>E. coli</i>	LH57	human	Laos	colistin	<i>mcr-1</i>	6	0.125	0.417	1.56	[20]
<i>E. coli</i>	1R4	human	Saudi Arabia	colistin	<i>mcr-1</i>	4	0.19	0.208	0.8	[21]
<i>E. coli</i>	NCTC 13846	human	England	colistin	<i>mcr-1</i>	4	0.19	0.208	0.8	NCTC
<i>E. coli</i>	6R	human	Saudi Arabia	colistin	<i>mcr-1</i>	4	0.125	0.417	1.56	[21]
<i>E. coli</i>	SE65	human	Algeria	colistin	<i>mcr-1</i>	8	0.19	1.67	6.25	[22]
<i>E. coli</i>	LH257	human	Laos	colistin	<i>mcr-1</i>	12	0.19	0.104	0.4	[20]
<i>E. coli</i>	134R	human	Saudi Arabia	colistin	<i>mcr-1</i>	3	0.19	0.208	0.8	[21]
<i>E. coli</i>	44A	human	Saudi Arabia	colistin	<i>mcr-1</i>	4	0.19	0.104	0.4	[21]
<i>E. coli</i>	TH99	human	Thailand	colistin	<i>mcr-1</i>	4	0.125	0.104	0.4	[20]
<i>E. coli</i>	235	chicken	Algeria	colistin	<i>mcr-1</i>	4	0.125	0.208	0.8	[20]
<i>E. coli</i>	CMUL64	human	Lebanon	carbapenem	<i>bla<sub>oxa-48</sub></i>	0.38	0.023	0.052	0.2	[23]
<i>E. coli</i>	CSURP5142	human	Marseille	carbapenem	<i>bla<sub>oxa-48</sub></i>	1	32	0.208	0.8	Unpublished data
<i>E. coli</i>	CSURP1954	human	Algeria	carbapenem	<i>bla<sub>NDM-5</sub></i>	0.25	> 32	0.052	0.2	[24]
<i>Klebsiella pneumoniae</i>	ATCC 13883	human	unknown	none	susceptible	1	0.38	0.052	0.2	ATCC
<i>K. pneumoniae</i>	LH70	human	Laos	colistin	unknown	12	0.19	0.052	0.2	[25]

<i>K. pneumoniae</i>	LH12	human	Laos	colistin	<i>mgrB</i>	32	0.19	0.104	0.4	[25]
<i>K. pneumoniae</i>	FHM169	human	France	colistin	<i>mgrB</i>	8	0.19	0.208	0.8	[25]
<i>K. pneumoniae</i>	TH20	human	Thailand	colistin	<i>mgrB</i>	32	0.19	0.417	1.56	[25]
<i>K. pneumoniae</i>	TH28	human	Thailand	colistin	<i>mgrB</i>	8	0.19	0.208	0.8	[25]
<i>K. pneumoniae</i>	TH176	human	Thailand	colistin	<i>mgrB</i>	12	0.19	< 0.052	< 0.2	[25]
<i>K. pneumoniae</i>	LH17	human	Laos	colistin	<i>mcr-1</i> + PmrB T157P	12	0.125	0.835	3.125	[25]
<i>K. pneumoniae</i>	LH92	human	Laos	colistin	<i>mcr-1</i>	12	0.19	0.104	0.4	[25]
<i>K. pneumoniae</i>	FHA60	human	France	colistin	<i>mcr-1</i>	8	0.19	0.104	0.4	[25]
<i>K. pneumoniae</i>	FHM128	human	France	colistin	<i>mcr-1</i>	4	0.19	0.417	1.56	[25]
<i>K. pneumoniae</i>	TH68	human	Thailand	colistin	<i>mcr-1</i>	8	0.19	0.104	0.4	[25]
<i>K. pneumoniae</i>	CSURP5123	human	France	carbapenem	<i>bla<sub>oxa-48</sub></i>	1	6	0.104	0.4	Unpublished data
<i>K. pneumoniae</i>	CSURP5233	human	France	carbapenem	<i>bla<sub>oxa-48</sub></i>	0.25	2	0.835	3.125	Unpublished data
<i>K. pneumoniae</i>	NCTC 13443	human	unknown	carbapenem	<i>bla<sub>NDM-1</sub></i>	1	> 32	0.208	0.8	Unpublished data
<i>K. pneumoniae</i>	CSURP5141	human	France	carbapenem	<i>bla<sub>NDM-1</sub></i>	0.25	4	0.835	3.125	Unpublished data
<i>K. pneumoniae</i>	CSUR5135	human	France	carbapenem	<i>bla<sub>NDM-1</sub></i>	1	0.38	0.417	1.56	Unpublished data
<i>K. pneumoniae</i>	CSURP1572	human	Algeria	carbapenem	<i>bla<sub>KPC-3</sub></i>	0.125	8	0.104	0.4	[26]
<i>K. pneumoniae</i>	853	human	Israel	carbapenem colistin	<i>bla<sub>KPC</sub></i> <i>mgrB</i>	48	> 32	0.104	0.4	Unpublished data
<i>K. pneumoniae</i>	1348	human	Israel	carbapenem	<i>bla<sub>KPC</sub></i>	< 2	8	0.835	3.125	Unpublished data
<i>K. pneumoniae</i>	695	human	Israel	carbapenem	<i>bla<sub>KPC</sub></i>	< 2	8	0.417	1.56	Unpublished data
<i>K. pneumoniae</i>	473	human	Israel	carbapenem	<i>bla<sub>KPC</sub></i>	< 2	6	0.417	1.56	Unpublished data
<i>Staphylococcus aureus</i>	CSURP1943	human	France	MRSA		-	-	26.27	< 100	[27]

<i>S. aureus</i>	ATCC 25923	unknown	unknown	none	susceptible	-	-	26.27	< 100	ATCC
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MRSA: Methicillin-resistant *Staphylococcus aureus*.

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## **2. Article 5. *Inactivation of thymidine kinase as a cause of resistance to zidovudine in clinical isolates of Escherichia coli: a phenotypic and genomic study - Peyclit L., Ben Khedher M., Zerrouki L., Diene SM., Baron SA., et Rolain J-M.***

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L'étude d'un mécanisme de résistance d'une molécule est importante afin de comprendre comment la bactérie s'adapte face au stress de la pression sélective. Il est plus aisé ensuite de prévenir et limiter son émergence. Chaque bactérie peut muter pour éliminer des cibles d'action des molécules auxquelles elle est exposée, mais le type et le taux de mutation varient selon les antibiotiques<sup>53</sup>. Après avoir suggéré l'usage de l'AZT en thérapie de dernier recours contre les entérobactéries résistantes, nous avons choisi d'étudier la résistance à l'AZT chez les entérobactéries ciblées. L'étude de la thymidine kinase, enzyme nécessaire à l'activation de l'AZT et potentiellement impliquée dans le mécanisme de résistance<sup>47,49</sup>, a été privilégiée.

Dans cet article, nous avons déterminé dans un premier temps la prévalence de la résistance à l'AZT à partir de 537 *E. coli* cliniques de notre hôpital. Nous avons ensuite étudié les séquences de la thymidine kinase des souches cliniques résistantes ainsi que la diversité de cette enzyme via les séquences de 633 génomes d'*E. coli* issus de la base de données PubMed. Enfin, le génome entier d'un mutant in vitro à l'AZT a permis d'explorer les variations par rapport à celui de la souche initiale sensible.

Après une première phase de dépistage sur une gélose Mueller-Hinton contenant 2,7 mg/l d'AZT, suivi de la réalisation de la CMI à l'AZT en milieu liquide, nous avons identifié 9 souches présentant une CMI à l'AZT supérieure à 26,7 mg/l. N'ayant pas de données officielles quant au seuil de sensibilité sur ce composé, nous avons donc considéré ces 9 isolats résistants en tenant compte des données cliniques et de la répartition des CMI de l'AZT chez ces espèces. Le gène de la thymidine kinase de ces souches a été séquencé. Il était absent dans 3/9 isolats, inactivé par des séquences d'insertions (IS) dans 2/9 isolats et muté dans 4/9 souches. L'analyse génomique de 633 génomes d'*E. coli* a montré l'hétérogénéité de la séquence du gène, avec 27 séquences différentes. Cependant, nous ne connaissons pas les CMI de ses souches. Nous avons ensuite créé in vitro un mutant résistant à l'AZT d'après une souche sensible *E. coli* : après séquençage du génome entier, celui-ci arborait 27 SNP (polymorphisme nucléotidique) dans 8 gènes du core-genome, comparé à la souche initiale. Cependant, la séquence de la thymidine kinase ne présentait aucune mutation : cette enzyme ne semble donc pas être la seule cible impliquée dans la résistance.

Face au nombre de souches résistantes trouvées et à la diversité de la thymidine kinase essentielle à l'activation de l'AZT, son utilisation en combinaison avec d'autres antibiotiques doit être privilégiée, de même que le contrôle au préalable de la sensibilité de la souche à traiter par une mesure de la CMI à l'AZT.

## Inactivation of thymidine kinase as a cause of resistance to zidovudine in clinical isolates of *Escherichia coli*: a phenotypic and genomic study

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**Objectives:** The antiviral zidovudine has been recently identified as an active drug against resistant Enterobacteriaceae, but prevalence of resistance to this compound remains unknown. The aim was to estimate the prevalence of clinical *Escherichia coli* isolates resistant to zidovudine and to decipher the mechanism of zidovudine resistance.

**Methods:** We screened 537 isolates on zidovudine-containing agar plates and studied their thymidine kinase (*tdk*) gene sequences, the putative target involved in zidovudine resistance. Moreover, sequence analysis of 633 complete genomes of *E. coli* was performed to investigate mutation in the *tdk* gene. A comparative genomic analysis was done on an *in vitro* zidovudine-resistant mutant.

**Results:** After screening on our medium containing 2.7 mg/L (10 µM) zidovudine, nine strains had a zidovudine MIC >26.7 mg/L. The gene was absent in three isolates, inactivated by an IS (IS1X2 and ISAp1) in two isolates and mutated in four isolates. A genomic analysis of 633 *E. coli* genomes showed heterogeneity of the *tdk* gene sequence, with 27 different sequences. Among them, three genomes showed an inactivation of the gene (IS, stop codon and no *tdk* gene sequence). The *in vitro* mutant *E. coli* had 27 SNPs in eight genes of the core genome compared with the initial strain.

**Conclusions:** Our study reports zidovudine-resistant clinical isolates of *E. coli*, presumably related to *tdk* inactivation. Diversity of Tdk in bacterial genomes can be large. Other mechanisms need to be considered in zidovudine resistance. The use of zidovudine in antibiotic-resistant infections needs to be in combination and should be tested before clinical administration.

### Introduction

The widespread attention given to resistance to last-line antibiotics,<sup>1</sup> the emergence of new resistance mechanisms<sup>2</sup> and the decrease in the development of new antibiotics<sup>3</sup> have led to the investigation of new therapeutic strategies.<sup>2</sup> An approved drug for a new therapeutic indication may be an alternative solution; this is called drug repositioning.<sup>3</sup>

We previously screened *in vitro* a panel of 1280 molecules approved by the FDA<sup>4</sup> against MDR Gram-negative bacteria. Zidovudine, an antiviral drug used in HIV therapy, was identified as an active molecule against carbapenem- and colistin-resistant Enterobacteriaceae.<sup>5–7</sup> Regarding its mechanism of action, thymidine kinase (Tdk) performs the first phosphorylation of zidovudine, essential to obtain the active form of the molecule.<sup>7</sup> As early as

1990, Lewin *et al.*<sup>8</sup> reported resistance to zidovudine by loss of Tdk activity. In 2011, Doléans-Jordheim *et al.*<sup>9</sup> observed inactivation of the *tdk* gene sequence by an IS in two *in vitro* zidovudine-resistant mutant clones of *Escherichia coli*, but the prevalence in clinical strains remains unknown. According to these initial studies, this enzyme seems to be involved in the resistance to zidovudine.

Since zidovudine has been proposed as a salvage therapy, alone or in combination, for the treatment of MDR bacterial infections,<sup>5</sup> our study aimed to determine the prevalence of zidovudine resistance in clinical strains using a large collection of *E. coli*. Bioinformatic analysis of 633 available complete genomes of *E. coli* was performed to investigate the inactivation of *tdk*. Finally, we analysed a susceptible *E. coli* and its zidovudine-resistant *in vitro* mutant.

## Materials and methods

Five hundred and thirty-seven *E. coli* were randomly selected from the clinical microbiology laboratory of the IHU-Méditerranée Infection, Marseille, France.

LB agar (Thermo Fisher Scientific, Waltham, MA, USA) containing zidovudine (Sigma–Aldrich, St Louis, MO, USA) was used as a screening medium at 2.7 mg/L (10 µM), the concentration used for our previous library screening. A spot of 10<sup>4</sup> bacteria was deposited on the agar and incubated for 18–24 h at 37°C. The results were interpreted visually; strains that grew on both LB agar and screening medium (LB agar + zidovudine) were considered resistant to zidovudine exposure. MIC was then determined using the broth microdilution method.

Molecular analysis was performed on strains with an MIC higher than 26.7 mg/L (100 µM). Using specific primers (F: GCAAGGCTTCGTAAGGGAGA; R: TATAGCCCGAAGAAGTGCG) we amplified and sequenced the *tdk* gene (Table S1, available as Supplementary data at JAC Online). The sequences obtained were then compared with the sequence from *E. coli* K12 (ABD37701). Further analysis was performed using PROVEAN and ISfinder.

In parallel, the Tdk protein reference sequence (ABD37701.1) from *E. coli* strain K-12 was used as a query to investigate its presence in all available complete genomes of *E. coli* from the NCBI database. A phylogenetic tree was constructed using the neighbour-joining method after protein alignment with MUSCLE. Mauve aligner and Easyfig v2.2.3 software were used to investigate the genetic environment of the *tdk* gene from three selected genomes.

In parallel, a zidovudine-resistant mutant was obtained after subculture of a susceptible strain, *E. coli* P4965, from our collection. Antimicrobial susceptibility testing, MIC determination for zidovudine and WGS using MiSeq (Illumina Inc., San Diego, CA, USA) technology were performed on these two strains.

## Results

### Susceptibility to zidovudine in clinical strains

Thirty-six of the 537 clinical strains (6.7%) grew on the agar medium containing 2.7 mg/L zidovudine (Figure 1a). Among these 36 strains, 9 had a zidovudine MIC >26.7 mg/L when tested using the broth microdilution method, as shown in Figure 1(b) and Table S2.

Of these nine strains, three (*E. coli* AL266, AL341 and AL430) were negative for amplification of the *tdk* gene, suggesting the loss of that gene. After amplification and sequencing of the remaining six isolates, four strains (*E. coli* AL5, AL172, AL237 and AL445) showed mutations: K15N, S101P and L116S were qualified as deleterious by PROVEAN, whereas Q191E was neutral (Figure 1c). Two ISs were identified in other amplicons: an IS1X2 of the IS1 family for *E. coli* AL342 and ISAp1 of the IS30 family for *E. coli* AL441.

### Bioinformatic analysis of complete genomes

The *in silico* analysis showed that, among the 633 complete *E. coli* genomes, 630 *tdk* sequences (99.53% of analysed genomes) exhibited an identity value ranging from 98.54% to 100% and 100% alignment with the reference sequence. In this dataset, 27 different sequences of the Tdk protein were identified. Gene 1 (Figure 2a) was the most frequent identified sequence, found in 215 genomes (34.13%). Phylogenetic tree analysis was performed with these 27 Tdk types and the four Tdk sequences identified in our clinical isolates. Using the Tdk sequence from our zidovudine-susceptible *E. coli* P4965 as reference, 11 amino acid substitutions

were identified (0.48%), considered deleterious by PROVEAN. Neutral mutations S74A (*n* = 29), Q191E (*n* = 14) and G193D (*n* = 5) were the most prevalent (Figure 2a).

Moreover, only 3 out of the 633 analysed genomes exhibited a Tdk sequence with low identity and coverage (from 31.03% to 39.58%) with the reference sequence. Genome analysis confirmed the absence of the *tdk* gene in one genome. For the two remaining genomes, the genomic environment was conserved but the *tdk* gene was altered (Figure 2b).

### Comparison between a susceptible strain and its resistant clone

*E. coli* P7345 was obtained from *E. coli* P4965 after one passage on the screening medium. For zidovudine, MIC values were >26.7 and 0.1 mg/L, respectively. The antibiotic susceptibility profile was the same in both strains for all the antibiotics tested according to EUCAST breakpoints (Table S3). The Tdk protein in the resistant clone had 100% identity and alignment compared with the initial strain. Comparative genome analysis showed an average nucleotide identity score of 99.9% and 27 SNPs present in eight genes of the core genome (Table S4).

## Discussion

In drug repurposing, zidovudine has been recently proposed as a promising candidate for colistin- or carbapenem-resistant bacterial infections.<sup>5</sup> Through this work, we estimated a prevalence of zidovudine resistance of 6.7% within *E. coli* clinical strains in Marseille, France, which represents a significant level of resistance. This resistance seems to be natural/intrinsic since these patients did not receive zidovudine therapy (Table S2).

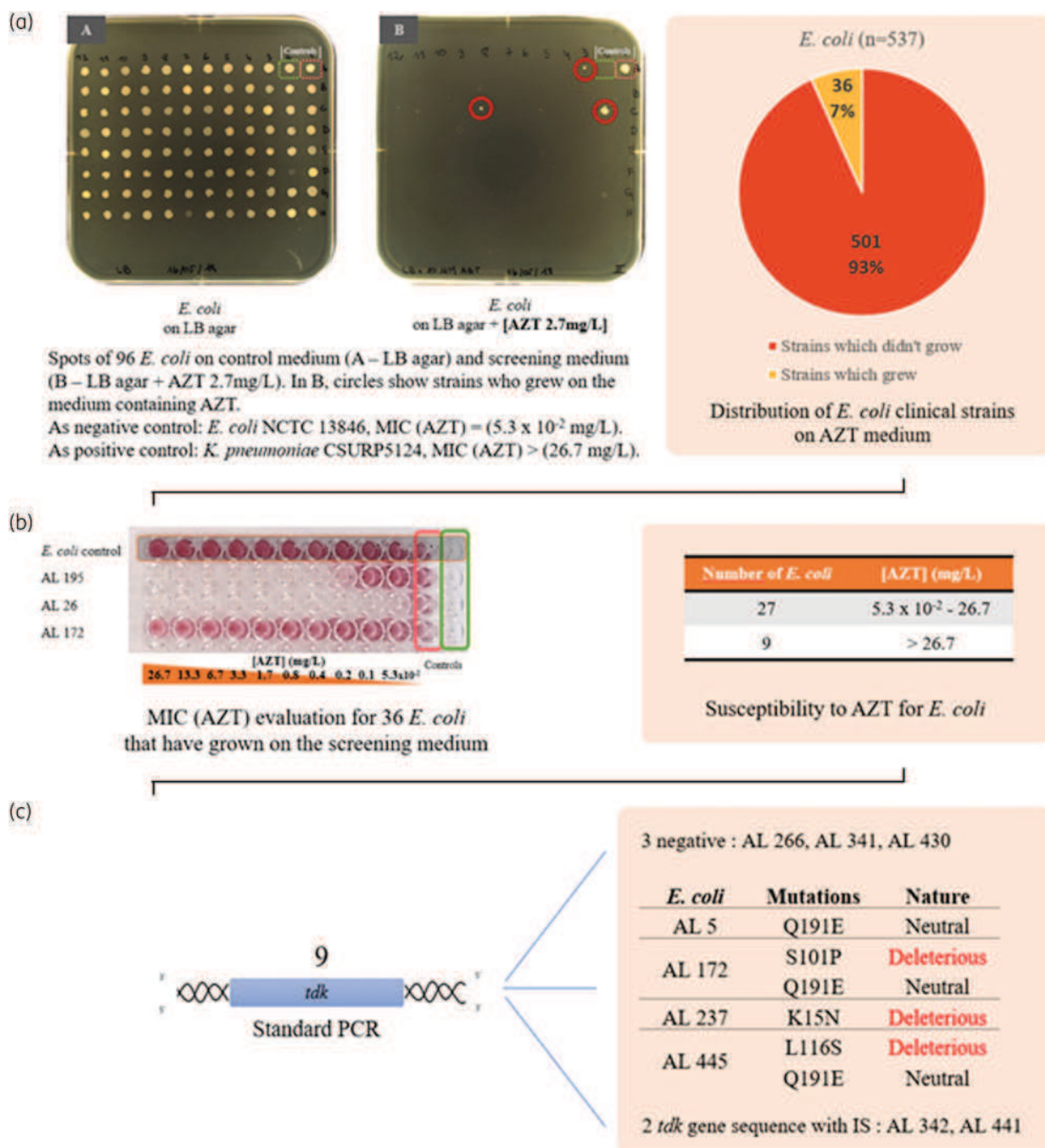
Zidovudine resistance has been shown *in vitro* to occur under zidovudine pressure, resulting in a higher mutation frequency.<sup>9</sup> The risk of selection is therefore significant, as Lewin et al.<sup>10</sup> had isolated zidovudine-resistant *E. coli* in zidovudine-treated AIDS patients.

We focused our molecular resistance investigation on Tdk, the first enzyme involved in zidovudine phosphorylation, which has already been described in *in vitro* zidovudine-resistant mutants.<sup>8,9</sup> Sequence analysis of the *tdk* gene in our clinical resistant strains confirmed that *tdk* could be impacted in zidovudine-resistant *E. coli*. Two deleterious amino acid modifications (S101P and L116S), the absence of the gene or truncation by IS1X2 and ISAp1 have not been described so far to be associated with zidovudine resistance. Thus, these modifications in *tdk* sequences could be involved in this resistance, considering that some of the mutations found had been described before<sup>9</sup> and that the inactivation of genes by IS is a common cause of resistance.<sup>11</sup>

Among the 633 *E. coli* genomes we found considerable diversity of *tdk* gene sequences. The *tdk* gene sequences 1, 2 and 3 are well represented and the sequence of *E. coli* K12 is only found in its genome. However, we do not know the susceptibility of these strains to zidovudine, which could have provided us with more information on the resistance mechanism.

Although the Tdk enzyme is involved in DNA synthesis,<sup>12</sup> there is wide sequence variability in *E. coli* or other species<sup>12</sup> and sometimes even an absence that does not affect bacterial growth. It appears that Tdk activity varies<sup>12</sup> according to its location, the





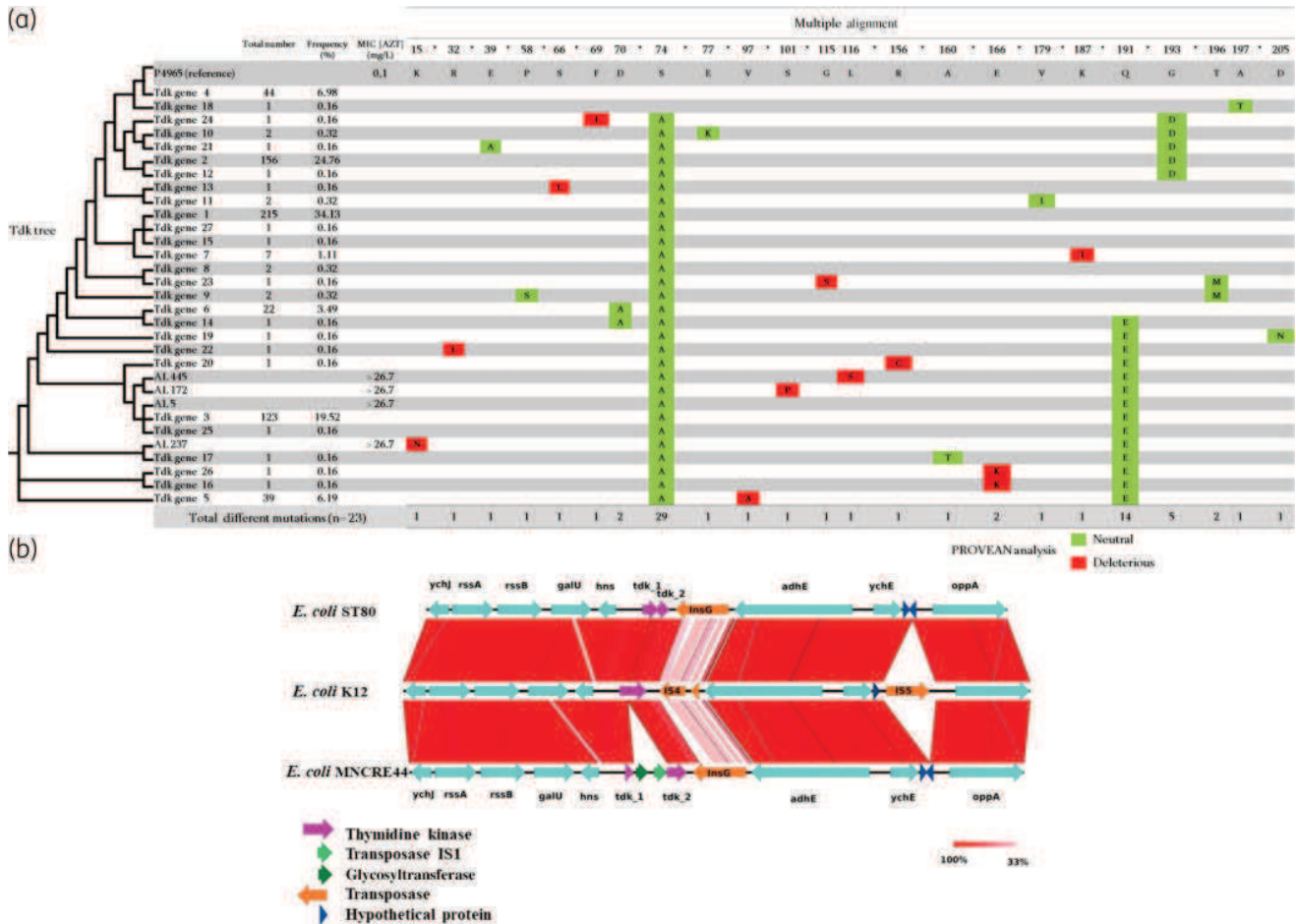
**Figure 1.** Global analysis of zidovudine exposure on clinical *E. coli* strains. (a) Assessment of clinical *E. coli* strains on a zidovudine screening medium. (b) Determination of zidovudine MIC values for strains that grew and identification of zidovudine-resistant strains. (c) Standard PCR on the *tdk* gene of resistant strains and molecular analysis of sequences. AZT, zidovudine. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

influence of other enzymes<sup>13</sup> and its level of methylation.<sup>14</sup> Thus, many other factors may therefore alter zidovudine activation<sup>7</sup> and thus its efficacy against *E. coli*.

Our study confirmed that resistance under zidovudine pressure can occur in an unmutated Tdk mutant. We identified eight

mutated genes, including an *rclA* gene involved in nucleotide binding and a gene encoding a hypothetical protein.

The use of zidovudine in clinics can lead to resistant strains but this may be limited by synergistic combinations.<sup>3</sup> Among antibiotics tested,<sup>9</sup> colistin has a synergistic relationship with zidovudine



**Figure 2.** (a) Distribution and mutations of all *tdk* gene sequences from available *E. coli* complete genomes and our clinical zidovudine-resistant strains. (b) Genetic environment analysis of the 14 kb surrounding the *tdk* gene in *E. coli* ST80 and MNCRE44 compared with *E. coli* K12. The genomic structure is almost the same except that the *tdk* gene from *E. coli* Es\_ST80 was split because of mutation leading to a stop codon, whereas that of *E. coli* MNCRE44 was disrupted by an IS1 family transposase and a glycosyltransferase gene. AZT, zidovudine. This figure appears in colour in the on-line version of *JAC* and in black and white in the print version of *JAC*.

against ESBL-, NDM-1- or MCR-1-producing Enterobacteriaceae isolates.<sup>15</sup> With a good pharmacological profile of safety,<sup>3</sup> no cross-resistance<sup>9</sup> and a clinical trial in progress,<sup>16</sup> zidovudine appears to be a promising candidate for drug repurposing in the treatment of MDR bacterial infections.

However, our study highlights that zidovudine-resistant *E. coli* are not uncommon, and therefore zidovudine susceptibility testing by MIC determination should be performed if clinicians wish to use this drug in combination to control bacterial infections.

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### Transparency declarations

None to declare.

### Author contributions

L.P., S.A.B., S.M.D. and J.-M.R. designed the study and drafted and revised the manuscript. L.P., M.B.K. and L.Z. performed microbiology analyses. All authors read and approved the final manuscript.

## Supplementary data

Tables S1 to S4 are available as Supplementary data at JAC Online

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### **3. Article 6. *Colistin and zidovudine, a synergic combination to fight clinical colistin-resistant K. pneumoniae* - Peyclit L., Baron SA., Rolain JM., et Li J.**

En cours de rédaction

Lors de la découverte et du développement des antimicrobiens, les modèles pharmacocinétiques/pharmacodynamiques (PK/PD) sont essentiels et limitent les différences entre la sensibilité *in vitro* de la molécule et son efficacité en clinique<sup>54</sup>. Ils permettent d'établir des informations de base sur l'interaction entre la bactérie et l'antibiotique. Ils aident ainsi à prédire les profils pharmacologiques de ces molécules pour leur usage en clinique. Les résultats obtenus à partir de modèles PK/PD *in vitro* ont par ailleurs montré de bonnes corrélations avec les données humaines et animales<sup>54</sup>. Bien qu'aucune composante immunitaire ne soit représentée dans ces modèles, ils se concentrent directement sur l'activité des antibiotiques eux-mêmes en l'absence de défenses de l'hôte, pouvant imiter alors le cas des patients immunodéprimés<sup>54</sup>.

Ces dernières années, la résistance à la colistine a augmenté dans le monde entier<sup>55</sup>. En raison de la plasticité génomique des isolats de *K. pneumoniae*<sup>56</sup> et de sa néphrotoxicité, l'utilisation de la colistine semble plus adaptée en association<sup>57</sup>. Son association avec des molécules non antibiotiques élargit les champs thérapeutiques pour lutter contre les bactéries « difficiles à traiter »<sup>58,59</sup>. Ayant précédemment démontré l'efficacité *in vitro* de l'AZT contre des souches de *K. pneumoniae* résistantes à la colistine<sup>60</sup>, nous avons également suggéré son usage dans le cadre d'une combinaison avec la colistine. Des études antérieures ont évalué l'efficacité de cette association contre des souches de *K. pneumoniae* résistantes à la colistine ou aux carbapénèmes<sup>61,62</sup>. Parmi des isolats *K. pneumoniae* résistants à la colistine de Grèce, cette combinaison était synergique dans 79 % des isolats et additive dans les 21 % restants<sup>62</sup>.

Le clone ST307 de *K. pneumoniae* apparu dans les années 2000 est majoritairement retrouvé en milieu hospitalier et dans l'environnement proche des hôpitaux<sup>63</sup>. Il est souvent associé à une résistance aux antibiotiques, notamment aux céphalosporines de 3<sup>ème</sup> génération et aux carbapénèmes, et à un risque élevé de propagation, causant notamment des épidémies intra-hospitalières<sup>64</sup>. La résistance à la colistine a également été observée chez ce clone d'où la suggestion d'une combinaison synergique.

Lin Y-W et coauteurs ont réalisé une étude préclinique de PK/PD sur l'association polymyxine B et zidovudine contre des souches de *K. pneumoniae* résistantes aux carbapénèmes<sup>65</sup>. C'est ainsi que lors d'un projet de collaboration avec cette même équipe, j'ai pu effectuer un projet de PK/PD à leurs côtés. Nous avons ainsi choisi de nous concentrer sur l'association colistine et AZT. Nous avons réalisé ici une étude statique avec un *time-kill* et un modèle PK/PD *in vitro* à un compartiment de cette association

contre deux isolats de *K. pneumoniae* ST307 résistants aux carbapénèmes, l'un étant aussi résistant à la colistine.

## Méthodes

### Sélection et sensibilité aux antibiotiques des souches

Les souches de *K. pneumoniae* 14CR<sup>66</sup> et *K. pneumoniae* KP\_DC<sup>67</sup>, isolées à partir d'échantillons cliniques de l'hôpital de Marseille, France appartiennent au clone multi-résistant ST307. Elles sont résistantes aux carbapénèmes (portant les gènes *OXA-48* et/ou *NDM-1*) et la souche 14CR est résistante à la colistine.

Les CMI de la colistine et de la zidovudine ont été déterminées par microdilution dans un bouillon Mueller-Hinton ajusté aux cations (CAMHB, Oxoïd, Hampshire, England). Les seuils de sensibilité pour la colistine sont définis à  $\leq 2$  µg/ml selon l'EUCAST en revanche aucune donnée n'a été fixé pour la zidovudine jusqu'à présent.

Le sulfate de colistine (lot n°20120719, Beta-Pharma, Princeton, États-Unis) et la zidovudine (lot n°LRAA9088, Merck KGaA, Darmstadt, Allemagne) ont été préparés en utilisant de l'eau « Milli-Q » à chaque début d'expérience.

### Expérience *time-kill*

Tous les tests ont été réalisés avec un inoculum initial de  $\sim 10^6$  CFU/ml dans 20 ml de CAMHB. La colistine, l'AZT et l'association ont été testées à différentes concentrations en fonction des CMI des isolats (de 0,5 à 8 fois la CMI). Des échantillons ont été prélevés à 0, 0,5, 1, 2, 4, 6 et 24 h, lavés avec une solution saline à 0,9 % puis centrifugés à  $3\,400 \times g$  à 37 °C pendant 10 minutes afin de minimiser la quantité d'antibiotique. Ensuite, ils ont étéensemencés sur des géloses nutritives pour un comptage viable des bactéries après 24 h d'incubation à 37°C.

### Modèle *in vitro* de PK/PD à un seul compartiment

Ce modèle a été réalisé sur la souche 14CR, résistante à la colistine, sur une durée de 48 heures. Des tests ont été effectués soit avec la colistine en perfusion continue et l'administration en bolus d'AZT toutes les 6 heures ou soit en perfusion continue pour les deux médicaments. La colistine a été ajoutée dans le réservoir de dilution pour atteindre une  $C_{\max}$  de 3 ou 1,5 mg/l dans le réservoir central et de la même manière, la zidovudine pour une  $C_{\max}$  de 4 mg/l. Lorsque l'AZT était administrée par injection bolus, une pompe à seringue automatisée (New Era Pump Systems, NY, USA) était connectée au réservoir central pour atteindre une  $C_{\max}$  de 4mg/l. Les mêmes schémas posologiques ont été simulés pour la combinaison. Deux colonies de cet isolat ont été évaluées dans le test où la concentration de colistine était réduite car nous disposions d'un réservoir supplémentaire. Différents réservoirs ont été utilisés selon le test : un réservoir témoin sans antibiotique, un pour la colistine en monothérapie, un pour la zidovudine en



monothérapie en perfusion continue, un avec la combinaison en perfusion continue, chacun contenant 80 ml de CAMHB à 37°C. Des échantillons ont été prélevés dans les réservoirs à 0, 1, 2, 4, 6, 23, 24, 25, 26, 28, 30, 47 et 48 h pour un comptage bactérien et une analyse de pharmacodynamique. Après avoir été lavés avec une solution saline à 0,9 % et centrifugés à  $3\,400 \times g$  à 37 °C pendant 10 minutes, les échantillons ont été ensemencés sur des géloses nutritives et incubés pendant 24 h à 37 °C. Les concentrations de colistine et d'AZT dans les réservoirs ont été mesurées a posteriori par CLHP (Chromatographie Liquide Haute Performance).

## Résultats

La souche de *K. pneumoniae* KP\_DC, co-productrice des deux enzymes NDM-1 et OXA-48, était résistante aux carbapénèmes<sup>67</sup> mais restait sensible à la colistine avec une CMI à 0,125 µg/ml comme précédemment rapporté. La souche de *K. pneumoniae* 14CR<sup>66</sup> était résistante aux carbapénèmes par sécrétion d'une OXA-48 et résistante à la colistine avec une CMI à 16 µg/ml. La CMI de la zidovudine était pour ces souches de 2 et 8 µg/ml respectivement (**Figures 1, 2**).

### Résultats du *time-kill*

L'AZT en monothérapie a entraîné une mort bactérienne en 2 heures avec une diminution de la concentration bactérienne de 3 log<sub>10</sub> pour des concentrations d'AZT comprises entre 1 mg/l et 16 mg/l chez *K. pneumoniae* KP\_DC (**Figure 1**). Le même effet a été observé pour *K. pneumoniae* 14CR en 4 heures avec une diminution de 2 log<sub>10</sub> pour des concentrations entre 4 mg/l et 32 mg/l (**Figure 2**). Aucun effet dépendant de la concentration n'a été observé. Une croissance (> 4 log<sub>10</sub>) similaire au groupe témoin a été observée pour toutes les concentrations dans les deux souches testées après 24 heures.

Pour la souche sensible *K. pneumoniae* KP\_DC, la colistine en monothérapie a montré un effet bactéricide en 2 heures pour toutes les concentrations démontrant ainsi une réduction bactérienne proportionnelle à la dose d'antibiotique. Une réduction de 5 log<sub>10</sub> a été observée à 0,5 mg/l (4xMIC) (**Figure 1**). Pour l'isolat résistant 14CR, toutes les concentrations de colistine (de 4 mg/l à 32 mg/l) ont montré une réduction bactérienne de  $\geq 2$  log<sub>10</sub> après 4h de traitement suivi d'une repousse majeure après 24h (**Figure 2**). À 24 heures, les concentrations bactériennes étaient similaires entre le groupe traité et le groupe témoin pour les deux isolats.

L'association de colistine et d'AZT contre *K. pneumoniae* KP\_DC a montré une mort bactérienne importante et rapide en moins de 2h avec une réduction de > 4log<sub>10</sub> pour toutes les concentrations testées. Une repousse est apparue après 6h pour toutes les concentrations. Contre *K. pneumoniae* 14CR, l'association a également montré une réduction bactérienne de > 5log<sub>10</sub> en 4 à 6 heures. Elle était suivie d'une repousse après 24h, excepté pour deux concentrations colistine/AZT : 32/8 mg/l et 64/8 mg/l après 24h (2/1xMIC, 4/1xMIC) (**Figure 2**). Après 24 heures, aucune des concentrations testées pour les deux

isolats présentait une concentration bactérienne similaire à celle du groupe témoin. Il y a donc un retard de la croissance bactérienne avec la combinaison par rapport aux monothérapies, dû à un effet synergique des deux molécules testées.

### **Cinétique du modèle PK/PD sur la souche *K. pneumoniae* 14CR (Figure 3)**

La colistine en perfusion continue en combinaison avec la zidovudine en injection bolus toutes les 6 heures a montré une très faible diminution des comptes bactériens et rapidement, toutes les concentrations bactériennes ont été similaires au groupe contrôle.

Les résultats sur la perfusion continue étaient plus convaincants. Les monothérapies ont permis de tuer les bactéries ( $\sim 2 \log_{10}$ ) en 6 heures, mais ont montré une repousse rapide pour tous les dosages après 24 heures. La combinaison colistine/AZT à 3/4 mg/l a montré une réduction synergique de  $4 \log_{10}$  du nombre de bactéries jusqu'à 24 heures puis une repousse à  $4 \log_{10}$  CFU/ml jusqu'à la fin de l'expérience. La combinaison à 1,5/4 mg/l a entraîné une mortalité bactérienne importante avec une réduction de  $6 \log_{10}$  pour la colonie 2 et de  $5 \log_{10}$  pour la colonie 1. Malgré cet effet, les deux colonies étaient à  $\sim 3 \log_{10}$  CFU/ml à 24h. A 48h, la colonie 2 a repoussé à la même concentration bactérienne que le groupe contrôle alors que la colonie 1 était à  $\sim 4 \log_{10}$  CFU/ml.

Les cinétiques des concentrations de deux molécules dans les réservoirs pendant les expériences sont en cours de réalisation par CLHP, ils nous permettront de valider les résultats précédents.

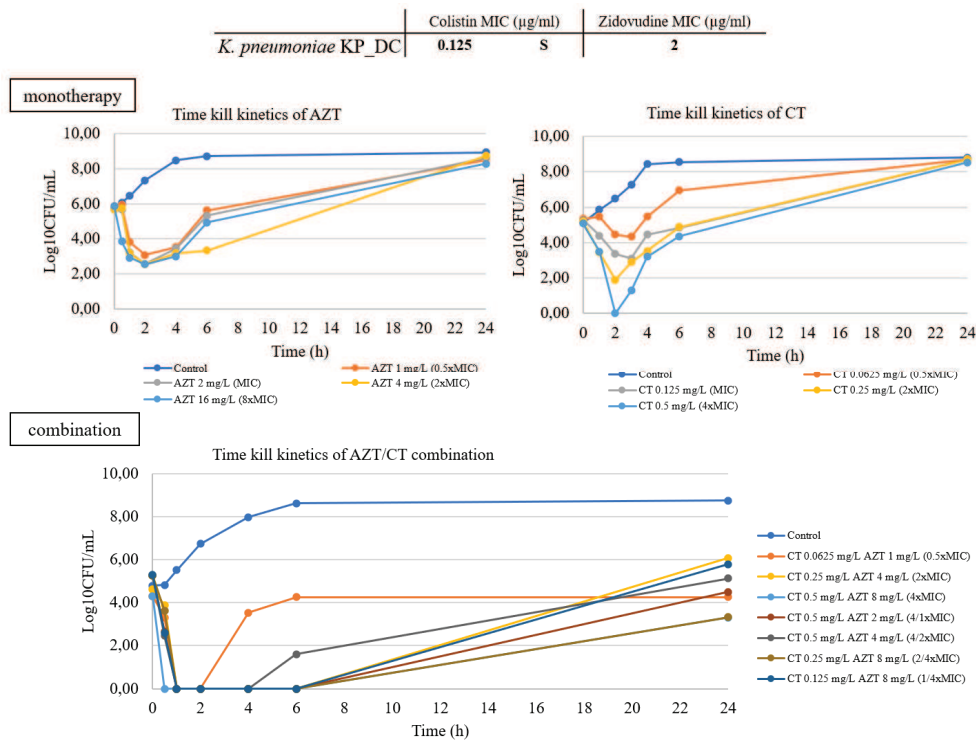
### **Conclusion**

L'effet de la zidovudine en monothérapie n'a pas été concluant au vue des données du *time-kill* et du modèle PK/PD, ceci nous confirme alors la nécessité de son utilisation en association. Nous avons démontré l'effet synergique in vitro de l'association colistine et AZT contre des souches *K. pneumoniae* résistantes aux carbapénèmes et à la colistine appartenant au clone ST307. Les concentrations de colistine et d'AZT utilisées dans le modèle in vitro sont cliniquement atteignables chez les patients<sup>68-70</sup>. Lin Y-W et coauteurs ont réalisé une modélisation PK/PD basée sur les mécanismes de la polymyxine B et l'AZT et ont conclu que la synergie était d'ordre mécanique : la polymyxine B augmente la concentration intracellulaire de la zidovudine par perméabilisation<sup>65</sup>. On peut alors supposer que le mécanisme est similaire pour notre association, la colistine agissant de la même façon que la polymyxine B.

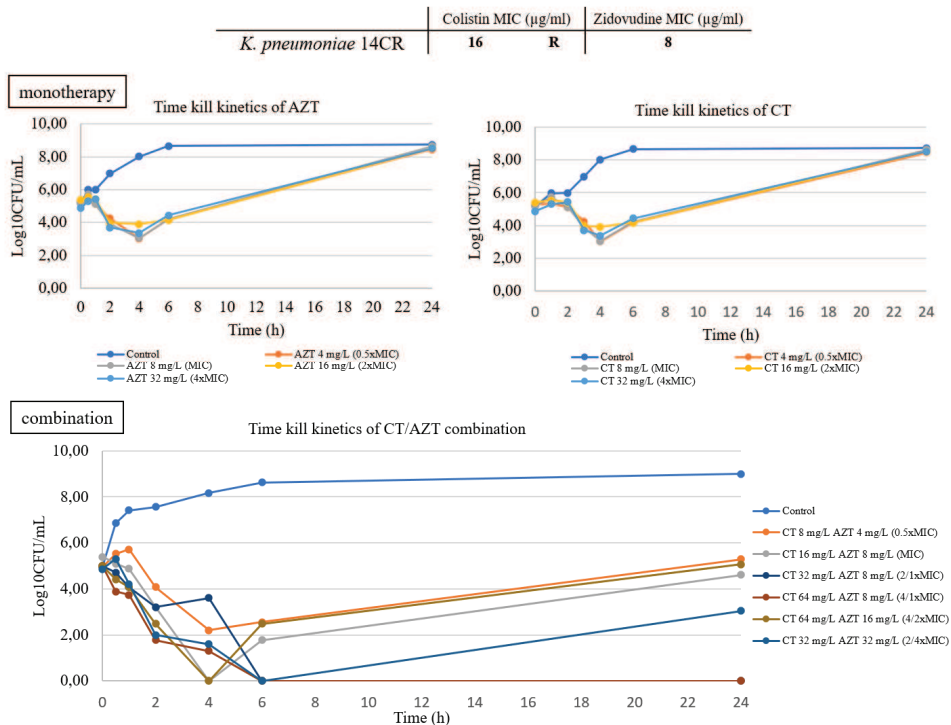
L'utilisation en perfusion continue de la combinaison semblait être plus efficace sur notre modèle in vitro que des injections bolus de zidovudine. Ces travaux doivent être poursuivis permettant de tester d'autres isolats et d'orienter vers des concentrations optimales.



## Figures



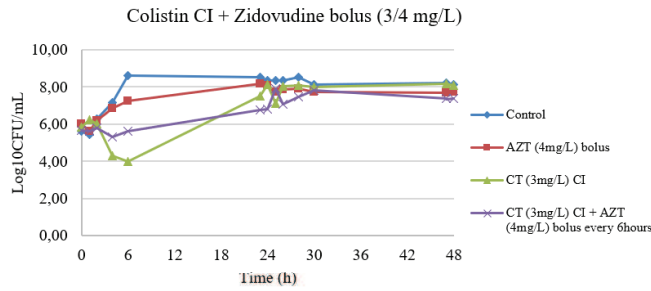
**Figure 1.** Cinétique de l’AZT, de la colistine et de la combinaison colistine/AZT sur un isolat sensible de *K. pneumoniae* (KP\_DC). CT : colistine, AZT : zidovudine



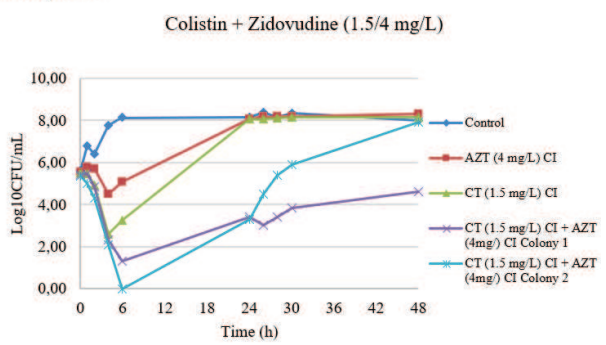
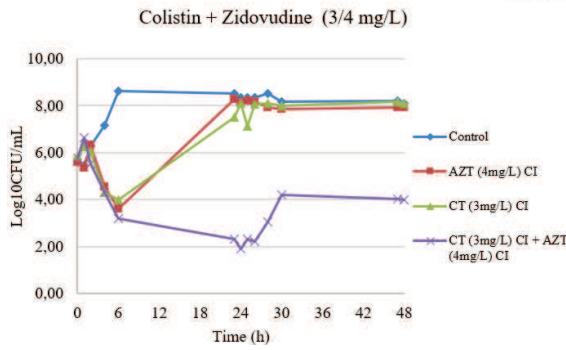
**Figure 2.** Cinétique de l’AZT, de la colistine et de l'association colistine/AZT sur un isolat de *K. pneumoniae* résistant à la colistine (14CR). CT : colistine, AZT : zidovudine

	Colistin MIC ( $\mu\text{g/ml}$ )		Zidovudine MIC ( $\mu\text{g/ml}$ )
<i>K. pneumoniae</i> 14CR	16	R	8

IVM kinetics



Continuous infusion



**Figure 3.** Cinétique du modèle PK/PD in vitro à un compartiment de l'association colistine/AZT sur la souche de *K. pneumoniae* 14CR, résistant à la colistine. CT : colistine, AZT : zidovudine, IVM : modèle in vitro

## 4. Conclusion

Après avoir étudié l'efficacité *in vitro* de la zidovudine, nous concluons qu'elle peut cibler efficacement des entérobactéries multi-résistantes qui peuvent poser un problème en pratique clinique. Toutefois, pour agir, elle doit être préalablement activée par la thymidine kinase. Cette enzyme, essentielle et conservée, peut être inactivée par des mutations, insertions ou délétions, ce qui nous amène donc à envisager son administration avec précaution. Nous suggérons son application comme agent antibactérien et donc la possibilité d'utilisation contre les entérobactéries multi-résistantes, en dernier recours, dans le cadre d'une combinaison avec les polymyxines.

L'AZT présente un potentiel thérapeutique contre les infections chez l'homme. De nombreuses études ont montré que l'AZT avait une puissante activité *in vivo*<sup>43</sup> : sur des modèles murins ou des veaux, l'AZT contrôlait des infections à *E. coli*, *K. pneumoniae* résistantes ou aux salmonelles<sup>43</sup>. Des études ont été menées pour améliorer davantage l'activité bactéricide de l'AZT et résoudre sa courte demi-vie via la création de dérivés. Ils pourraient être particulièrement utiles contre les infections bactériennes opportunistes pour des patients atteints du SIDA<sup>43</sup>.

Les travaux préliminaires de Hu Y. et coauteurs sur la combinaison colistine et AZT semble avoir été les prémisses d'essais cliniques<sup>61</sup>. La société Helperby Therapeutics Group Ltd qui a apporté le support financier de cette étude, lance en 2020 la phase de 2 de l'essai clinique de Zidistin®, formé de colistine et d'AZT, contre des infections urinaires compliquées et projette un essai clinique pour le même composé dans le traitement d'infections intra-abdominales<sup>71,72</sup>. Des tests *in vitro*, *in silico* et *in vivo* sont encore nécessaires, notamment pour définir la relation entre la sensibilité *in vitro* et l'efficacité *in vivo* de l'AZT pour une bactérie.

## VI. Conclusion générale

La résistance aux antibiotiques s'est propagée à travers le monde et dans divers milieux, facilitée par l'utilisation des antibiotiques par l'homme en médecine humaine ou de manière intensive dans l'élevage animal. Dans les infections humaines, la résistance aux molécules utilisées en dernier recours (les carbapénèmes ou des vieilles molécules comme la colistine) compromet le traitement des patients infectés à l'hôpital. Malgré certains discours alarmistes, peu de bactéries sont extrêmement résistantes à tous les antibiotiques mais dans certains cas, les cliniciens font face à des infections difficiles à traiter. Surveiller et caractériser ces résistances semble essentiel pour adopter la meilleure thérapeutique possible contre ces bactéries multi-résistantes.

Nous avons vu tout au long de cette thèse que peu de nouvelles molécules anti-infectieuses avaient été mises à disposition des praticiens au cours de ces dernières années. Les grandes entreprises pharmaceutiques s'investissent moins dans le développement des antibiotiques, n'y trouvant pas un marché lucratif<sup>73</sup>. Cependant, d'autres solutions thérapeutiques sont aujourd'hui disponibles ou en cours de développement. Dans ce projet, le repositionnement moléculaire a permis d'étudier la sensibilité des bactéries, multi-résistantes et pathogènes pour l'homme, à un grand nombre de molécules que les cliniciens ont à leur portée, et notamment des molécules indiquées pour d'autres cibles ou pathologies. Cette technique offre des solutions rapides et peu toxiques. Nous avons pu voir concrètement que cette alternative thérapeutique apportait des solutions prometteuses, pour les bactéries résistantes aux médicaments de dernier recours comme avec la zidovudine, mais aussi pour des bactéries opportunistes présentant des résistances intrinsèques. Une solution précise et spécifique ne peut pas constituer un remède à ces infections multi-résistantes : les mécanismes de résistance sont en perpétuelle évolution et varient en fonction des pays, des hôpitaux et des espèces bactériennes. Les antibiotiques ne doivent plus être considérés comme l'arme ultime contre ces bactéries. Avoir notion d'un panel d'options variées comme la combinaison de molécules synergiques, le repositionnement médicamenteux ou l'augmentation des doses lorsque cela est tolérable est primordial. Toutefois, nous avons pris conscience que certaines de ces molécules ne sont pas communes dans le traitement des infections comme les anticancéreux et que leur utilisation peut être freinée par les cliniciens par manque de données d'efficacité. Avant leur administration, ces options sont à vérifier au laboratoire dès lors que le profil de résistance bactérien est évalué. Ces solutions doivent ainsi être adaptées à chaque infection, à chaque patient comme une médecine personnalisée en tenant compte des paramètres du pathogène mis en cause mais aussi des comorbidités du patient : c'est le principe de la balance bénéfice/risque.

Repositionner une molécule permet d'éviter la phase 1 des essais cliniques qui consiste à étudier les paramètres pharmacocinétiques et toxiques de la molécule sur des volontaires sains. L'efficacité de la zidovudine a pu être évaluée par des études in vitro sur des entérobactéries résistantes. De plus, la tolérance chez l'homme est connue de longue date puisqu'elle est largement utilisée dans le traitement du VIH. Sa mise à disposition dans le traitement des infections à bactéries résistantes pourrait voir le jour rapidement avec la phase 2 qui est en cours. Par ailleurs, l'évaluation de la prévalence de sa résistance nous a confirmé la nécessité de son utilisation en combinaison.

La recherche sur la résistance aux antibiotiques permet d'informer et d'aider la communauté médicale sur les potentiels risques liés à une explosion ou une démocratisation de cette multi-résistance. L'utilisation de nouveaux outils comme le séquençage des génomes bactériens ouvrent de nouvelles perspectives dans la surveillance ainsi que dans la compréhension des nouveaux mécanismes de résistance. Dans le futur, ces outils bio-informatiques pourraient aussi rentrer dans le cadre d'une médecine adaptée en étant une alternative aux antibiogrammes et aux diagnostics cliniques.

En définitive, nos écosystèmes sont complexes et évolutifs et selon la théorie d'Alice et le croquet vivant<sup>74</sup>, les prédictions ne permettent pas d'anticiper l'émergence des épidémies ou des résistances bactériennes : chaque paramètre impliqué peut changer à tout moment, rendant le résultat attendu totalement différent<sup>74</sup>. Des solutions de secours doivent toujours être recherchées pour éviter un jour des impasses thérapeutiques.

## VII. Perspectives

Les nouvelles découvertes de mécanismes de résistance aux antibiotiques et la persistance d'infections difficile à traiter en clinique nous confirment que ce sujet est toujours d'actualité. Des projets de recherche doivent être maintenus pour limiter ces phénomènes et les contrôler. Ce travail a permis de mieux comprendre et appliquer la stratégie du repositionnement moléculaire pour traiter les bactéries multirésistantes. Cependant, plusieurs pistes sont encore à approfondir.

Dans le cadre du projet sur la zidovudine, il nous semble évident d'évaluer la prévalence de souches cliniques résistantes à la combinaison en développement, colistine et zidovudine. Pour cela, le seuil de sensibilité à l'AZT des souches doit aussi être établi pour l'interprétation des CMI. Nous souhaiterions également poursuivre la compréhension du mécanisme de résistance à la zidovudine de certaines souches où la thymidine kinase est inchangée, par une complémentation du gène ou des analyses transcriptomiques.

Concernant les résultats trouvés dans le criblage des 1280 molécules contre les bactéries multi-résistantes, nous aimerions continuer d'évaluer les autres hits les plus pertinents par des tests *in vitro* et sur un plus grand nombre de souches. De là, nous envisageons la même stratégie que nous avons opté pour la zidovudine afin d'avancer vers le repositionnement moléculaire de nouvelles molécules.

Enfin, nous aimerions développer un projet dans notre institut pour mettre en place à plus grande échelle ce criblage moléculaire lorsque les cliniciens font face à des bactéries difficiles à traiter.

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## IX. Autres travaux

La surveillance et l'étude des BMR est primordiale à la fois pour détecter les mécanismes d'action nouveaux mais aussi pour évaluer leur impact. Nous pouvons alors améliorer les stratégies de prévention et de traitement et ainsi mieux contrôler et limiter les conséquences non désirées des infections aux BMR. C'est dans cette optique que deux de mes travaux suivants ont été réalisés.

Lors de mon master 2 effectué dans le même laboratoire, j'ai pu collecter plus de 250 souches de différentes espèces et effectuer leur sensibilité aux différents antibiotiques ; cette collection de bactérie m'a été utile ensuite durant mes trois années d'internat d'IPR permettant de sélectionner des souches pour nos expériences. Les souches ont été répertoriées grâce à toutes les études épidémiologiques réalisées auparavant par notre équipe. Ces études servent à la surveillance de l'émergence de bactéries résistantes et l'étude de leur mécanisme d'action. Nous les avons rapporté dans l'**article 7** intitulé « *Role of Institut Hospitalo-Universitaire Méditerranée Infection in the surveillance of resistance to antibiotics and training of students in the Mediterranean basin and in African countries* » (Peyclit L, Chanteloup A, Hadjadj L, Rolain J-M. *New Microbes New Infect.* 2018;26. doi:10.1016/j.nmni.2018.06.001). Les isolats y sont très variés et proviennent de l'environnement, des animaux et de l'homme. Les prélèvements sont issus de divers pays du bassin méditerranéen et contiennent une grande diversité de mécanismes de résistance aux antibiotiques.

Par ailleurs, lors de mon internat, j'ai pu effectuer pendant deux semestres une collaboration avec le Professeur Jian LI, responsable du laboratoire de pharmacologie des systèmes antimicrobiens à l'institut de découverte de la biomédecine de Monash University, Melbourne, Australie. Nous voulions bénéficier de leur expertise en pharmacodynamie et pharmacocinétique pour étudier la combinaison zidovudine et colistine mais également pour pouvoir travailler avec eux sur l'étude des mécanismes de résistance à la colistine. Nous avons étudié des souches cliniques de *K. pneumoniae* appartenant au clone ST307, résistantes à la colistine. Aucune de ces souches ne portait le gène *mcr*. Nous avons choisi de comparer ces souches par analyse génomique avec le séquençage de leur génome mais aussi via la structure de leur lipide A, fraction hydrophobe du LPS qui permet son ancrage à la membrane externe de la bactérie et qui est associé à la résistance à la colistine. Les résultats ont été retranscrits dans l'**article 8** : « *Comparative genomics and lipid A profiling of ST307 Klebsiella pneumoniae strains from Marseille, France with various colistin susceptibility* » (Peyclit L., Han M., Baron S., Rolain J-M., et Li J. En cours de rédaction.)



« *SCA medium: a new culture medium for isolating all Candida auris clades* » (Ahmad Ibrahim, **Lucie Peyclit**, Hanane Yousfi, Rim Abdallah, Jean-Marc Rolain et Fadi Bittar. En cours de rédaction. Journal of clinical microbiology)

Congrès scientifiques :

- **Peyclit L.**, Baron S., Yousfi H., and Rolain J.-M. Zidovudine: a salvage therapy for *mcr-1* plasmid-mediated colistin-resistant bacterial infections? (poster) **11 Octobre 2018**, Journée de la recherche, Faculté de Pharmacie, Marseille, France
  - **Peyclit L.**, Baron S., Rolain J.-M. Evaluation du potentiel antibactérien de molécules sur des bactéries multi résistantes. (poster) **17-18 Décembre 2018**, 38<sup>ème</sup> RICAI, Paris, France
  - **Peyclit L.**, Zerrouki L., Baron S., Rolain J.-M. Prevalence and resistance to zidovudine in Enterobacteriaceae clinical strains due to inactivation of thymidine kinase. (poster) **13-16 Avril 2019**, 29<sup>th</sup> ECCMID (Société Européenne de Microbiologie Clinique et Maladies Infectieuses), Amsterdam, Pays-Bas
  - **Peyclit L.**, Ben Khedher M., Zerrouki L., Baron S., Rolain J.-M. Inactivation of thymidine kinase as a cause of resistance to zidovudine in clinical strains of *Escherichia coli*: a phenotypic and genomic study. (poster) **6-9 Novembre 2019**, 31<sup>st</sup> ICC (Société internationale de chimiothérapie antimicrobienne) - 4th GCCMID, Dubaï, Emirats Arabes Unis
  - **Peyclit L.**, Baron S., Rolain J.-M. Identification of the Anti-bacterial Potential of Drugs on Multidrug-resistant Bacteria Among 1280 FDA-approved Drugs.
- Peyclit L.**, Han M., Baron S., Rolain J.-M., Li J. Comparative Genomics and Lipid A Profiling of ST307 *Klebsiella Pneumoniae* Strains from Marseille, France with Various Colistin Susceptibility. (posters) **18-22 Juin 2020**, ASM Microbe 2020, Chicago, Illinois (e-posters, suite à l'épidémie du COVID-19)