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Antibiotic combination therapy can select for broad-spectrum multidrug resistance in *Pseudomonas aeruginosa*

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ABSTRACT

Combination therapy with several antibiotics is one strategy that has been applied in order to limit the spread of antimicrobial resistance. We compared the de novo evolution of resistance during combination therapy with the β-lactam ceftazidime and the fluoroquinolone ciprofloxacin with the resistance evolved after single-drug exposure. Combination therapy selected for mutants that displayed broad-spectrum resistance, and a major resistance mechanism was mutational inactivation of the repressor gene *mexR* that regulates the multidrug efflux operon *mexAB–oprM*. Deregulation of this operon led to a broad-spectrum resistance phenotype that decreased susceptibility to the combination of drugs applied during selection as well as to unrelated antibiotic classes. Mutants isolated after single-drug exposure displayed narrow-spectrum resistance and carried mutations in the MexCD–OprJ efflux pump regulator gene *nfxB* conferring ciprofloxacin resistance, or in the gene encoding the non-essential penicillin-binding protein DacB conferring ceftazidime resistance. Reconstruction of resistance mutations by allelic replacement and in vitro fitness assays revealed that in contrast to single antibiotic use, combination therapy consistently selected for mutants with enhanced fitness expressing broad-spectrum resistance mechanisms.

1. Introduction

Combination therapy with two or more drugs is the standard treatment for infections with human immunodeficiency virus (HIV), *Plasmodium falciparum*, *Mycobacterium tuberculosis* and, in cystic fibrosis (CF) patients, *Pseudomonas aeruginosa* [1–4]. There are multiple rationales for combination therapy. The clinical considerations include therapeutically covering the spectrum of potential pathogens during polymicrobial infections or in acute infections for which the responsible micro-organism or resistance profile of the pathogen is unknown [5]. Using different classes of antimicrobial drugs in combination might result in synergistic antibiotic interactions that enhance the inhibitory effect [5]. None the less, the general benefits of combination therapy compared with single or sequential administration of antibiotics for treating bacterial infections have been difficult to conclusively demonstrate [4]. Combination treatment might even have negative effects in infections with Gram-negative bacteria owing to increased treatment costs and increased risk of adverse side effects. However, since combination therapy could potentially lower the rate of de novo antibiotic resistance evolution, it is considered a potential resistance management strategy [4].

Theoretically, the probability of mutations conferring resistance to two agents simultaneously is lower than the probability of mutations conferring resistance to a single agent. However, this probability applies only if resistance mutations occur independently and drugs are administered simultaneously for enhanced effect. Considering the pharmacodynamic and pharmacokinetic complexity of multidrug treatment, reaching synchronised, therapeutically inhibitory concentrations at the site of infection in the clinical setting is problematic [2,6]. General resistance phenotypes caused by biofilm growth or persister state, or the potential selection for broad-spectrum resistance mechanisms activated via one-step mutational events confounds predictions about the potential overall benefit of combination therapy over monotherapy as a tool for controlling the spread of antibiotic resistance. We propose the following model by which bacterial populations acquire

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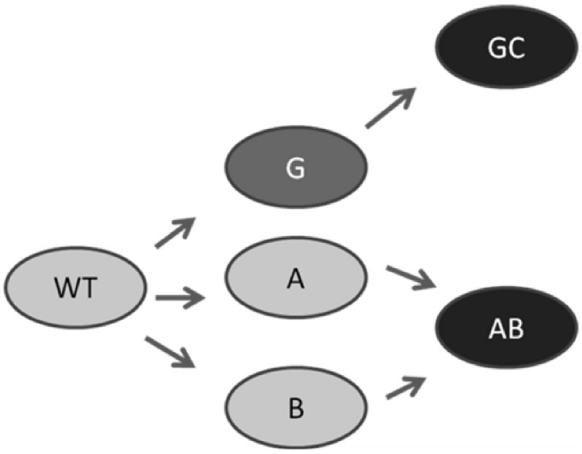

Fig. 1. Pathways for selecting general resistance mechanisms. A mutation or state that gives a general resistance phenotype has a selection advantage over specific resistance mutations when two drugs are present. 'A' (*dacB*) and 'B' (*nfxB*) confer resistance to drug 'a' (ceftazidime) and drug 'b' (ciprofloxacin), respectively; 'G' (*mexR* or unknown) confers a general resistance phenotype; 'C' represents a compensatory event that either increases resistance or reduces the fitness cost of G. The data from this study showed that G (*mexR* or unknown) can occur at the same frequency as A (*dacB*) or B (*nfxB*), increasing the probability of broad-spectrum resistance when two drugs are present. WT, wild-type.

broad-spectrum resistance during combination therapy. Resistance to antibiotics 'a' and 'b' is conferred by the resistance mutations 'A' and 'B', either through the acquisition of first A and then B, or first B and then A (Fig. 1). However, we hypothesise that in the presence of both drugs, a mutation or state that results in a general resistance phenotype ('G') towards a and b simultaneously confers a selective advantage compared with the specific resistance mutations (A or B) when the two drugs are present simultaneously. The G state does not necessarily need to provide a survival advantage compared with an A + B double mutant, but will have a fitness advantage compared with the A or B single mutant when both drugs are present. Moreover, the G state will have the advantage of requiring fewer additional mutations to reach full resistance and will therefore change the resistance evolution trajectory by potentiating the evolution of novel resistance traits, either acquisition of the specific resistance mutations a and b, which requires multiple mutations, or acquisition of an additional compensatory mutation 'c' that enhances G to levels of resistance comparable with resistance provided by A and B (Fig. 1). To understand the consequences of antibiotic combination therapy, we investigated the spectrum of resistance mutations that were selected during single-drug, sequential drug and combination therapy. In vitro experiments were performed challenging the Gram-negative human pathogen *P. aeruginosa* with a β-lactam drug ceftazidime that targets cell-wall synthesis, primarily penicillin-binding protein 3 (PBP3), and a fluoroquinolone ciprofloxacin that disrupts DNA replication by inhibiting the enzymes topoisomerase II and topoisomerase IV [7,8]. The antibiotics tested in the present study are used in the treatment of CF patients with *P. aeruginosa* lung infections, who are sometimes simultaneously treated with both drugs [3,9]. The molecular mechanisms underlying the resistance phenotypes were identified using whole-genome sequencing and allelic replacement. Each therapy selected for different resistance mechanisms, with combination therapy resulting in broad-spectrum resistance whereas antibiotic monotherapy selected for narrow-spectrum resistance. These results have important implications for combination therapy as a clinical tool for antibiotic resistance management.

2. Materials and methods

2.1. Strains, media, susceptibility testing and mutant selection

Derivatives of *P. aeruginosa* strain PAO1 were used in this study [10]. A complete list of strains and plasmids is presented in Table 1. All strains were grown at 37 °C in Luria–Bertani (LB) broth with aeration at 200 rpm with or without ciprofloxacin (Bayer HealthCare AG, Leverkusen, Germany) and/or ceftazidime (Sigma-Aldrich, St Louis, MO). Minimum inhibitory concentrations (MICs) were determined for ceftazidime, ciprofloxacin and meropenem using Etest strips (bioMérieux S.A., Marcy-l'Étoile, France) on LB agar plates at 37 °C for 22 h according to the manufacturer's guidelines. Etest MICs for combinations of ciprofloxacin and ceftazidime were determined according to the manufacturer's guidelines (Etest application sheet EAS 023; AB BIODISK, bioMérieux S.A.). Selection of resistant mutants was on LB agar plates containing antimicrobials at 5× MIC for PAO1 (4 mg/L ceftazidime and 1 mg/L ciprofloxacin for single-drug therapy, and 1.5 mg/L ceftazidime + 0.2 mg/L ciprofloxacin for combination therapy). Single-drug selections were also performed at 1.5 mg/L ceftazidime and 0.2 mg/L ciprofloxacin. Mutation frequencies were calculated as antibiotic-resistant CFU divided by total CFU determined on antibiotic-free Luria agar plates.

Table 1
Additional strains used in this study.

Strain	Relevant genotype/phenotype	Selecting agent (g/mL)	Source/reference
PAO1	*Pseudomonas aeruginosa* wild-type		[10]
MV146	*mexR*(G188A)		This study
MV148	*dacB*(G211A)		This study
MV176	*dacB*(G211A) + *nfxB*(C65T)		This study
MV182	*nfxB*(C65T)		This study
MV124		CIP 1	This study
MV125		CIP 1	This study
MV122		CAZ 4	This study
MV123		CAZ 4	This study
MV237		CIP 0.2 + CAZ 1.5	This study
MV132		CIP 0.2 + CAZ 1.5	This study
MV155		CIP 0.2 + CAZ 1.5	This study
MV152		CIP 0.2 + CAZ 1.5	This study
MV127		CAZ 4 → CIP 1	This study
MV128		CAZ 4 → CIP 1	This study
MV129		CAZ 4 → CIP 1	This study
CC118 λλpir	*Escherichia coli* lysogenised with *λpir* phage		[11]
pRK600	RK2-Mob$^+$ RK2-Tra$^+$, Cm^r		[12]
pNJ1	*sacB*, Tet^r, R6 K origin		[16]