Assessing the Emergence of Resistance: The Absence of Biological Cost *In Vivo* May Compromise Fosfomycin Treatments for *P. aeruginosa* Infections

Alexandro Rodríguez-Rojas¹,ª, María D. Maciá², Alejandro Couce¹, Cristina Gómez³, Alfredo Castañeda-García¹, Antonio Oliver², Jesús Blázquez¹

¹Centro Nacional de Biotecnología (CNB), Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain, ²Servicio de Microbiología and Unidad de Investigación, Hospital Son Dureta, Instituto Universitario de Investigación en Ciencias de la Salud (IUNICS), Palma de Mallorca, Spain, ³Servicio de Anatomía Patológica, Hospital Son Dureta, Palma de Mallorca, Spain

Abstract

**Background:** Fosfomycin is a cell wall inhibitor used efficiently to treat uncomplicated urinary tract and gastrointestinal infections. A very convenient feature of fosfomycin, among others, is that although the expected frequency of resistant mutants is high, the biological cost associated with mutation impedes an effective growth rate, and bacteria cannot offset the obstacles posed by host defenses or compete with sensitive bacteria. Due to the current scarcity of new antibiotics, fosfomycin has been proposed as an alternative treatment for other infections caused by a wide variety of bacteria, particularly *Pseudomonas aeruginosa*. However, whether fosfomycin resistance in *P. aeruginosa* provides a fitness cost still remains unknown.

**Principal Findings:** We herein present experimental evidence to show that fosfomycin resistance cannot only emerge easily during treatment, but that it is also cost-free for *P. aeruginosa*. We also tested if, as has been reported for other species such as *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*, fosfomycin resistant strains are somewhat compromised in their virulence. As concerns colonization, persistence, lung damage, and lethality, we found no differences between the fosfomycin resistant mutant and its sensitive parental strain. The probability of acquisition *in vitro* of resistance to the combination of fosfomycin with other antibiotics (tobramycin and imipenem) has also been studied. While the combination of fosfomycin with tobramycin makes improbable the emergence of resistance to both antibiotics when administered together, the combination of fosfomycin plus imipenem does not avoid the appearance of mutants resistant to both antibiotics.

**Conclusions:** We have reached the conclusion that the use of fosfomycin for *P. aeruginosa* infections, even in combined therapy, might not be as promising as expected. This study should encourage the scientific community to assess the *in vivo* cost of resistance for specific antibiotic-bacterial species combinations, and therefore avoid reaching universal conclusions from single model organisms.

Introduction

Amongst a number of old antibiotics being used once more as appealing alternatives for the treatment of different infections, fosfomycin (Fos) displays some features that place it as a very promising compound [1]. Although its use has traditionally been focused on urinary tract infection pathogens, fosfomycin is a broad-spectrum bactericidal antibiotic, that is active against both gram-positive and gram-negative bacteria [2]. It has shown almost no toxicity and allergenicity in humans [3]. It displays little cross-resistance with other antibiotics, probably because it is chemically unrelated to any other known antimicrobial agent [2,3]. Also, several studies have pointed out the synergistic effect of fosfomycin when used in combination with other antimicrobials [4,5,6,7]. Fosfomycin treatments have shown a relatively low proneness of resistant mutants to persist *in vivo*, leading to a good effectiveness in the therapy, at least against *Escherichia coli* [8]. However, recently the acquisition of fosfomycin resistance in a previously circulating CTX-M-15-producing *E. coli* O25b-ST131-phylogroup B2 strain has been reported. This apparently occurred when the use of fosfomycin was increased by 50% [9].

The emergence of antibiotic resistance in a bacterial population is shaped by several factors, of which the mutation rate to resistance and the fitness cost of the mutants have been considered critical [10]. If resistance implies a considerable cost for the bacteria, their growth rate would not be enough to offset the wash...
out dynamic imposed by the diverse body fluids, the killing mediated by the immune system, or it might allow the more fit susceptible bacteria to out-compete the resistant ones once the antibiotic is removed [10].

The trend of resistant bacteria to have a competitive disadvantage in an untreated environment is related to the precise mechanism of the resistance in question. Numerous types of resistance achieved by chromosomal mutations are caused by target alterations [11]. In these cases a consistent fitness cost may appear if the resistant target displays a somewhat suboptimal functionality, as has been shown, for example, with some mutations in the gene tspL conferring streptomycin in bacteria such as E. coli or Salmonella spp. [12]. Another prominent way to attain resistance is determined by accessory elements, where resistance is afforded by enzymes or pumps that inactivate or remove the antimicrobial from the cell [13]. This type of resistance may also give rise to fitness cost, as the replication and activity of the elements themselves could involve some metabolic modifications [21].

In the specific case of fosfomycin, resistance is mainly acquired by reducing the cell uptake of the antibiotic [14,15,16]. This kind of resistance has been widely shown to compromise E. coli at a slower rate of growth when compared to that of the sensitive parental strains [8,17,18], probably because most mutations disturb carbon metabolism [8]. In addition, and very conveniently for humans, these mutants also displayed a reduced virulence represented by lower adhesion to epithelial cells [18,19,20]. The data for other species are scarce, but it has been reported that Klebsiella pneumoniae and Proteus mirabilis, common bacteria also causing uncomplicated urinary tract infections, follow the same trend as E. coli [18]. Surprisingly, a new resistance mechanism based on amino acid substitutions in MurA, the target protein of fosfomycin, has recently been reported. Unfortunately, the authors have not estimated the cost associated to such a modification [21].

Recently it has been reported that the use of fosfomycin be extended in order to treat pathogens like Pseudomonas aeruginosa [2], where the emergence of antibiotic resistance has led to an unpleasant scarcity of treatments [22]. P. aeruginosa is one of the leading nosocomial pathogens worldwide. The most severe infections produced by P. aeruginosa occur in Intensive Care Unit patients, those suffering from chronic respiratory diseases and immunocompromised individuals [23]. Considering the example of E. coli, it seems reasonable to expect that fosfomycin might serve as a genuine alternative to treat infections caused by multidrug resistant P. aeruginosa. This possibility has been substantiated by published data showing that fosfomycin, furthermore, could reveal a particular effectiveness when used in combined therapy with other well-known antipseudomonal agents [2,4,5,6,7].

In a previous work, we reported that P. aeruginosa has a high mutation frequency to fosfomycin resistance in vitro [24]. These observations in vitro could undermine the good expectations created about fosfomycin. However, sometimes the data generated in vitro do not agree with the results attained in vivo [10,25]. Here, we undertook the assessment of this disturbing possibility, monitoring the emergence of fosfomycin resistant (Fos-R) mutants in an in vivo murine model. We then checked the fitness cost caused by the inactivation of glpT in one insertion (null) mutant, as well as its virulence relative to the sensitive parental strain. Our results indicate a deviation from the E. coli model, disproving any connection between fosfomycin resistance, fitness cost or loss of any trait conferring bacterial virulence in P. aeruginosa.

### Methods

#### Ethics Statement

All animal experiments were approved by the animal ethics committee of the University of the Balearic Islands, Spain. All animals were handled and housed in strict accordance with guidelines from the University of the Balearic Islands.

#### Bacteria and media

The P. aeruginosa PA14 and its derivative mutants mutS::MAR2xT7 and glpT::MAR2xT7 were kindly provided by Dr. N. T. Liberati [26]. All the P. aeruginosa strains were cultured in Luria-Bertani (LB) or Mueller-Hilton at 37°C, and gentamicin (10 µg/ml) was added when appropriate. For the biofilm assay, LB and FBA minimal media were used [27].

**P. aeruginosa lung infection mouse model**

The murine model of P. aeruginosa lung infection was established by intranasal inoculation of bacteria [28,29,30]. Briefly, for the preparation of the inocula, bacteria (P. aeruginosa strains PA14, PA14mutS::MAR2xT7, PA14glpT::MAR2xT7 or a 1:1 mix of PA14/PA14glpT::MAR2xT7) were grown in LB to 0.5 OD 600 nm, centrifuged at 10,000 g for two minutes and diluted 10-fold in saline solution [1 and 5-fold dilutions in saline solution were also tested during the standardization of the model]. Finally, serial 1/10 dilutions of bacterial suspensions were plated in MHA to estimate bacterial viability counts.

Female C57BL/6J mice were used. All animals weighed 20 to 25 g and were provided by Harlan Ibérica, S. L. The animals were specific pathogen free and nourished ad libitum with sterile water and food. Before inoculation, the mice were anesthetized by intraperitoneal injection of ketamine and xylazine (100 mg and 10 mg per kg of body weight respectively). Forty microliters of the corresponding bacterial suspension, containing the indicated bacterial counts, were inoculated intranasally by pipetting directly in nasal orifices.

In addition to the evaluation of associated mortality and lung bacterial load, lung histopathology studies were performed. Briefly, lungs were fixed in 10% formalin, embedded in paraffin, sectioned (4 to 6 µm), and stained with hematoxylin and eosin. Lung inflammation was then blindly scored by an expert pathologist using the criteria of Johansen et al. [31] (1, normal; 2, mild focal inflammation; 3, moderate to severe focal inflammation with areas of normal tissue; 4, severe inflammation to necrosis and severe inflammation throughout the lung).

#### Pulmonary infection survival model

Three groups of 10 mice each per strain were inoculated with two different bacterial inocula of 1×10⁶ and 5×10⁵ of bacteria per animal of strains PA14 and PA14glpT::MAR2xT7 approximately, and mortality was recorded for 7 days. A Kaplan-Meier test was performed to determine the difference between mortality curves in the survival experiments. P values less than 0.05 were considered to be statistically significant.

#### Estimation of spontaneous Fos-R mutation rates

For an estimation of the in vitro spontaneous mutation rate of PA14 and its derivative mutant mutS::MAR2xT7 strains, approximately 10⁸ cells from overnight cultures were inoculated in 8 tubes, each containing 1 ml of LB, and subsequently incubated at 37°C with strong shaking for 16 h. Aliquots from successive dilutions or concentrated cultures were plated onto Mueller-Hinton agar (MHA) plates with fosfomycin (128 µg/ml). Additionally, appropriate dilutions with no antibiotic were plated onto

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**Fosfomycin Fitness Cost**

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MHA to estimate viability. The number of colonies growing after 24 h of incubation were determined. The mutation rate was then estimated using the Jones median estimator [32], and 95% confidence intervals were calculated using JONATOR (v0.1), a software developed in gfortran which implements an algorithm described elsewhere [33]. This software is free and available upon request. The same procedure was followed for an estimation of the mutation rate in vivo, but in this case, 8 mice were inoculated intranasally with approximately 5 × 10⁵ bacteria per animal from log-phase cultures of PA14 and the mutS-deficient strain. Lung homogenates were then obtained 48 hours postinfection, as described elsewhere. Proper dilutions of the lung homogenate were plated, as described for the procedure in vitro.

Estimation of the frequencies of spontaneous mutants resistant to fosfomycin, tobramycin and imipenem

An estimation of the frequencies of spontaneous mutants resistant to fosfomycin (128 µg/ml), tobramycin (4 µg/ml), imipenem (4 µg/ml) and the combinations of tobramycin plus fosfomycin (4 and 128 µg/ml, respectively) and imipenem plus fosfomycin (4 and 128 µg/ml, respectively) was performed for the PA14 and mutS::MAR2xT strains, as previously described [24]. In the case of the glpT::MAR2xT strain, the mutant frequency was determined for tobramycin (4 µg/ml) and imipenem (4 µg/ml) to study the possible epistatic effects of resistance to fosfomycin and these two antibiotics.

Evaluation of the fitness and in vivo selection of fosfomycin resistant mutants

Mice were inoculated with approximately 5 × 10⁷ cells of strains PA14, PA14mutS::MAR2xT7 and PA14glpT::MAR2xT7 in saline solution. Twenty-four hours after inoculation, two groups of 8 mice per strain were either treated with fosfomycin by receiving two intraperitoneal injections in saline solution with an interval of 8 hours (200 mg/kg/8 h) [34,35,36] or untreated. After 48 hours of inoculation (16 h after the end of the treatment in the case of fosfomycin-treated mice), the animals were sacrificed and their lungs aseptically extracted. The left lung was homogenized in 2 ml of saline using the Ultra-Turrax T-25 disperser (IKA, Staufen, Germany). Serial 1/10 dilutions were plated on MHA and MHA with 10 µg/ml of tetracycline. The minimal inhibitory concentration (MIC) of fosfomycin was determined as described [39] for three colonies of every transformed mutant. The glpT gene of all mutants was sequenced, and compared with the wild type gene as described [37].

Biofilms

An abiotic solid surface biofilm formation assay was performed in 96-well polystyrene microtiter plates in LB and minimal FBA [27] media in a humid chamber at 37°C, as described previously [40]. Biofilms were quantified after seven days of incubation. Forty independent replicas were carried out for each strain. Data were compared by a Student’s t-test.

Antibiotic susceptibility testing

Minimal inhibitory concentrations (MICs) of fosfomycin, tobramycin and imipenem were determined for the PA14 strain and its derivative mutants mutS::MAR2xT7 and glpT::MAR2xT7 by the broth microdilution method, as recommended by the CLSI [39].

Results

glpT null mutant is as lethal as its parental strain

We did not detect any statistically significant difference in mortality between the PA14 wild type strain and the null mutant glpT::MAR2xT7 (Fos-R), independently of the size of the inoculum (Figure 1). The Kaplan-Meier analysis revealed no discrepancy in the mortality curves between strains receiving the same inoculum (p = 0.81, p = 0.21, for approximately 1 × 10⁶ and 5 × 10⁵ bacteria, respectively). These results indicate that the disruption of the glpT gene does not interfere with the infective capacity and mortality caused by P. aeruginosa in this model.

Effect of fosfomycin treatment in bacterial load of the wild type, Fos-R, and mutS strains

The colonization of mice lungs by P. aeruginosa PA14 (median 9.16 × 10⁵, interquartile range from 1.57 × 10⁵ to 1.73 × 10⁶) forty eight hours post infection did not show statistical differences in the number of colony-forming units (cfu) per mouse, in comparison with the other two strains, mutS::MAR2xT7 (median 8.86 × 10⁵, interquartile range from 2.15 × 10⁵ to 2.55 × 10⁶) and glpT::MAR2xT (median 2.95 × 10⁵, interquartile range from 1.12 × 10⁵ to 1.30 × 10⁵) (p = 0.44). On the other hand, when mice were treated with fosfomycin, bacterial counts of the wild type.
strain (median $6.02 \times 10^5$, interquartile range from $2.65 \times 10^5$ to $1.66 \times 10^5$) and the mutS-deficient mutant (median $6.60 \times 10^5$, interquartile range from $1.18 \times 10^5$ to $2.06 \times 10^5$) were dramatically decreased by two orders of magnitude ($p = 0.005$ and $p = 0.009$, respectively). However, the glpT::MAR2xT mutant did not suffer any drop in bacterial counts (median $4.19 \times 10^7$, interquartile range from $2.01 \times 10^7$ to $1.06 \times 10^8$) with no differences with the non-treated experiment ($p = 0.91$) (Figure 2A).

The proportion of resistant mutants is greatly increased in the wild type strain after fosfomycin treatment (Figure 2B), especially if we consider that this increase appeared only after 24 hours from the time the antibiotic was administered. The untreated group has the expected proportion of Fos-R mutants according to its mutation frequency (median $1.58 \times 10^{-2}$, interquartile range from $3.62 \times 10^{-7}$ to $5.69 \times 10^{-2}$). However, the proportion of wild type Fos-R cells increased by two orders of magnitude (median $9.82 \times 10^{-5}$, interquartile range from $1.36 \times 10^{-3}$ to $5.76 \times 10^{-5}$), which represents a more than 60 fold increase and is statistically significant ($p = 0.009$) (Table 1). Furthermore, for the PA14 mutS::MAR2xT7 strain this phenomenon was aggravated. While the proportion of resistant mutants in untreated mice was as expected (median $2.54 \times 10^{-4}$, interquartile range from $8.27 \times 10^{-5}$ to $7.71 \times 10^{-4}$), in treated mice the proportion increased up to two fosfomycin resistant mutants for every ten bacteria (median $1.70 \times 10^{-3}$, interquartile range from $2.71 \times 10^{-2}$ to $7.15 \times 10^{-3}$), which corresponds to a greater than 600 fold increase and is statistically significant ($p = 0.001$) (Table 1).

One interesting point is that the median of the absolute number of mutants actually remains constant if we compare the Fos-R mutant load in mice receiving fosfomycin or not, although the total bacterial count decreased by two orders of magnitude in the dosed mice. This indicates that from the start of fosfomycin dosing, probably because the bacterial load was very high at that point, there is a multiplicity problem that limits the amount of mutants. However, when mice are dosed, the number of fosfomycin-susceptible bacteria diminish, thus the resistant ones can prevail at a higher rate in comparison with the non-treated group.

**Figure 1. Mortality curves.** Mice were infected with two different inocula of P. aeruginosa PA14 (blue lines) and its mutant glpT::MAR2xT7 derivative (red lines) strains. The Kaplan-Meier analysis failed to detect any significant difference between curves from the same bacterial inoculum ($1 \times 10^5$ cell (crosses) and $5 \times 10^5$ cells (diamonds)) ($p = 0.81$ and $p = 0.21$, respectively). doi:10.1371/journal.pone.0010193.g001

**Figure 2. Recovery of bacteria from lungs 48 hours post infection.** Mice were infected with approximately $5 \times 10^5$ cfu/animal and treated with two doses of fosfomycin (200 mg/kg) 24 hours after inoculation or with no antibiotic. PA14 (blue columns) and its mutant derivatives mutS::MAR2xT7 (red columns) and glpT::MAR2xT7 (gray columns) (A). Ratio of Fos-R mutants/total bacteria in lungs from treated and non-treated mice (B). Values are medians and error bars represent interquartile ranges. doi:10.1371/journal.pone.0010193.g002
R mutants in vivo experiments indicate that there is no limitation in the emergence of in vivo experiments. The high dispersion in the data corresponding to the laboratory conditions of the variation of the animal models compared to the controlled 

Table 1. Amplification of fosfomycin resistant mutants in mice treated with the antibiotic.

| Strains       | FosR mutant/total cfu (treated mice) | FosR mutant/total cfu (untreated mice) | Fold increase in Fos-R |
|---------------|-------------------------------------|---------------------------------------|------------------------|
| PA14 WT       | 9.8x10^5                            | 1.58x10^5                             | 61.98                  |
| mutS::MAR2xT7 | 1.70x10^4                            | 2.54x10^4                             | 666.76                 |

Fold increase values were calculated as the ratio between the median of the proportion of Fos-R mutants/total bacterial counts (figure 2B) of treated and untreated mice.

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Mutation rates to fosfomycin resistance in vivo are similar to those in vitro

Results shown in figure 3 indicate that the mutation rates obtained in vitro and in vivo are very similar (95% confidence interval). As expected, mutation rates to fosfomycin resistance are in agreement with the previously described values for these P. aeruginosa strains [24].

Wild type strain did not outcompete the Fos-R mutant in vivo or in vitro

The in vitro competition experiment rendered no statistical differences between the WT strain and glpT mutant (CI = 1.04; p = 0.80). Similarly, in the in vivo competition experiment in mice which did not receive fosfomycin treatment, the competition index between the wild type strain and glpT-deficient mutant, was 0.78, indicating no fitness advantage in any strain (p = 0.22). As expected, the scenario changed totally when the mice were treated with fosfomycin. In this case the CI rose to 9.70 in favour of the Fos-R mutant (p = 0.02), showing that Fos-R mutants will prevail in the population when pressure is exerted by fosfomycin.

All Fos-R mutants generated in vivo contain mutations in the glpT gene

Complementation studies showed that glpT mutations were the cause of fosfomycin resistance in vivo in all cases. Ten randomly chosen mutants from the wild type strain recovered fosfomycin sensitivity upon complementation with the glpT wild type gene. These results coincide with those from our previous work [37].

Inactivation of glpT does not affect biofilm formation in vitro

We could not detect any statistically significant difference between the wild type strain and its glpT::MAR2xT7 derivative in biofilm formation. Figure 4 shows the ability of the two strains to form biofilm in FBA (mean ± standard deviation: 0.19±0.07 for the wild type and 0.17±0.05 for the glpT-deficient mutant, p = 0.31) or LB (mean ± standard deviation: 0.39±0.12 for the wild type and 0.41±0.13 for the glpT-deficient mutant, p = 0.59).

Histopathology studies

Inflammation was scored in 10 mice per strain by using the criteria of Johansen et al. [31]. In summary, both strains (PA14 and PA14/glpt::MAR2xT7) induced a very high level of inflammation (3.9±0.32 and 3.6±0.97; mean and standard deviation of scores for PA14 and PA14/glpt::MAR2xT7, respectively) with no significant differences between them (p = 0.40). Most of the infected mice (90% and 80% for PA14 and PA14/glpt::MAR2xT7, respectively) had a score of 4 (from severe inflammation to necrosis and severe inflammation throughout the lung) (Figure 5).

Frequency of emergence of resistant mutants to combined antibiotics

To investigate the possibility of appearance of double mutants with resistance to the combinations of fosfomycin with different antibiotics, we first determined the MICs of these antibiotics for the PA14 strain and its derivatives mutS::MAR2xT7 and glpT::MAR2xT7 (Table 3). The mutant frequencies of resistance to the combinations of tobramycin plus fosfomycin (4 and 128 µg/ml, respectively) and imipenem plus fosfomycin (4 and 128 µg/ml, respectively) were also studied. Obviously, the frequencies of mutants resistant to the single antibiotics fosfomycin (128 µg/ml), tobramycin (4 µg/ml) and imipenem (4 µg/ml) were also calculated. Table 4 shows that the frequency of mutants resistant to the combination of fosfomycin with tobramycin is below our limit of detection (≤10−11), making improbable the emergence of resistance to both antibiotics when administered together, even in a hypermutable background. However, the frequency of mutants resistant to the combination of imipenem plus fosfomycin is relatively high in the wild type (2.1x10−7) with a 2-log increase in the hypermutator mutant (1.1x10−7). These results suggest that
the generation of mutants resistant to the combination of fosfomycin with some antibiotics may not be very difficult for *P. aeruginosa*. Interestingly, the frequencies of mutants resistant to either tobramycin or imipenem of the *gltT*-deficient strain were similar to those of the wild type. Thus, there is apparently no epistatic effect between the resistance determinants of the pairs tobramycin/fosfomycin and imipenem/fosfomycin.

**Discussion**

Among the different factors contributing to the ascent of antibiotic resistance, fitness cost is now recognized as prominent and one that ought to be evaluated when assessing the suitability of any drug treatment [8,10]. Here we have shown that in the absence of fosfomycin, the *P. aeruginosa* PA14 wild type strain) after two days of *in vitro* or 24 hours of *in vivo* competition.

![Figure 4. Seven-day biofilm formation. Biofilm formation of *P. aeruginosa* PA14 (red columns) and its mutant derivative *gltT*:MAR2xT7 (gray columns) is shown. The values are represented as the mean ± SD. Forty replicas were performed per strain and medium. No statistically significant differences were detected between strains for each medium (FBA, p = 0.31; LB, p = 0.59).](image)

Similar results were obtained previously in an *in vitro* experiment comparing growth rate in minimal media [37].

On the other hand, after two days of acute lung infection, the bacterial lung counts and the ability of the *gltT* mutant to cause death did not differ from the parental strain. These results clearly indicate that fosfomycin resistance does not exert a significant burden on fitness or virulence for *P. aeruginosa*.

This divergence from the *E. coli* patterns could be explained to some extent by the fact that, although both species share the reduction in uptake of fosfomycin on the basis of resistance mechanism [8,14,15,16,37], they apparently differ in the specific target genes for mutation and their metabolic implications. It is well established that, in *E. coli* and other gram negative species fosfomycin access primarily through the L-alpha-glycerol-3-phosphate transport system (*gltT*), and to a lesser extent via the hexose phosphate transport system (*uhpT*) [15]. Moreover, these two genes are known to be positively regulated by cAMP, and so mutations that lower the cAMP level in the cell (i.e. in the *cya* and *ptsI* genes) can induce resistance as well [41]. This reduction in *cAMP* levels can produce profound alterations to the bacterial metabolism, and can probably explain the significant reduction in fitness and virulence shown for many resistant isolates in *E. coli* [8].

Aldoght, *Fos-R* clinical isolates of *E. coli* are now being found and suggesting that this is a viable threat [9,21].

In contrast, the uptake of fosfomycin in *P. aeruginosa* depends exclusively on GlpT permease [37], which does not seem to be regulated by *cAMP* levels. Indeed, the *gltT* gene is isolated in the *P. aeruginosa* genome (www.pseudomonas.com), while the *E. coli* ortholog is included inside the well-known *glt* operon [42]. This may be related to another radical difference between these two species, as has been stated in the case of *P. aeruginosa* which displays a higher preference for glycerol as carbon source than for glycerol-3-phosphate [43]. These observations could be understood considering the different ecology of both species.

We have also performed an analysis of the *gltT* sequences from *Fos-R* mutants obtained in *in vivo* and complementation studies, which show that fosfomycin resistance is acquired in *P. aeruginosa* by any mutation capable of inactivating GlpT activity. From a comparative point of view with *in vitro* generated mutants, mutational pathways appear to be very similar in both conditions.
Our results have at least two relevant implications from a medical standpoint. First, our data demonstrate that the fitness cost of antibiotic resistance needs to be measured for each particular species of interest, and that the observations made in just one species cannot be extrapolated to another, since the genetic particularities might modulate the effects of bacterial fitness. Moreover, fosfomycin resistant clinical isolates of E. coli are now being found, suggesting that this is a viable threat [9, 21]. Second, our results curb the good prospects that fosfomycin has recently promised, at least for its use on P. aeruginosa infections. Similarly, it has been reported that there is no repercussion for fitness in any other resistance mechanism mediated by gene inactivation in P. aeruginosa. A partial deregulation of ampC expression by mutation of its repressor ampD confers efficient resistance to cephalosporins, without biological cost, due to two other copies of ampD [44]. However, contrarily to what occurs with glpT mutations, where a very high level of resistance is conferred, the ampD-based resistance is far from being complete. The high proportion of fosfomycin mutants generated by the hypermutable mutS-deficient strain indicates a real danger of rapid emergence of resistant mutants during a treatment. This is especially true in chronic respiratory infections, where one of the most prevalent and dangerous bacterial pathogens is P. aeruginosa [45]. In these infections the prevalence of hypermutable strains is abnormally high [46] and have been linked to antibiotic resistance development and persistence in respiratory airways [47, 48]. Thus, one can expect that in such infections fosfomycin resistance would develop as soon as patients begin the treatment.

As fosfomycin has been proposed as potentially useful in combined therapy [49], we studied in vitro the probability of acquisition of resistance to the combination of fosfomycin with two different antibiotics, tobramycin and imipenem. While the combination of fosfomycin with tobramycin makes improbable the emergence of resistance to both antibiotics when administered together, even in a hypermutable background, the results from the combination of fosfomycin plus imipenem suggest that the generation of mutants resistant to both antibiotics is not very difficult for P. aeruginosa. However, additional studies are still needed to determine the real effectiveness of fosfomycin when administered together with these and other antibiotics. Recently Ward et al [50] have reported that the biological cost of resistance to a second antibiotic in P. aeruginosa could be greater than that of previously acquired resistance. Finally, the total cost depends on the genetic background in which resistance evolve and its interaction with the environment. Well conducted studies are needed to find a good combination of antibiotics where double resistance, if available, has a sufficiently high cost to impede the development of resistance.

| Table 3. Minimal inhibitory concentrations of the antibiotics fosfomycin, tobramycin and imipenem for PA14 and its derivative mutants mutS::MAR2xT7 and glpT::MAR2xT7. |
| --- |
| **Antibiotic** | **MIC (µg/ml)** |
| | **wt** | **mutS::MAR2xT7** | **glpT::MAR2xT7** |
| Fosfomycin | 8 | 8 | 1024 |
| Tobramycin | 0.25 | 0.25 | 0.25 |
| Imipenem | 0.5 | 0.5 | 0.5 |

Table 4. Frequency of mutants resistant to single antibiotics and the combinations of fosfomycin with tobramycin and imipenem.

| Antibiotic | Concentration (µg/ml) | Mutant frequency |
| --- | --- | --- |
| **MutS::MAR2xT7** | **wt** | **glpT::MAR2xT7** |
| Tobramycin | 2.2 x 10^{-6} | 1.4 x 10^{-7} |
| Imipenem | 2.3 x 10^{-6} | 9.1 x 10^{-4} |
| Fosfomycin | 1.5 x 10^{-6} | 1.3 x 10^{-4} |
| **Combination** | **wt** | **glpT::MAR2xT7** |
| Tobramycin + Fosfomycin | < 10^{-11} | < 10^{-11} |
| Imipenem + Fosfomycin | 2.1 x 10^{-9} | 1.1 x 10^{-7} |

a: antibiotic concentration in µg/ml.

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Antibiotherapy for the different pathogens nowadays threatening humankind.

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Author Contributions
Conceived and designed the experiments: ARR AO JB. Performed the experiments: ARR MDMA CG AC JB. Analyzed the data: ARR AC CG AO JB. Contributed reagents/materials/analysis tools: AO JB. Wrote the paper: ARR MDMA AC JB.

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