Role of inorganic phosphate concentrations in *in vitro* activity of fosfomycin

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**A B S T R A C T**

Objectives: The objective of this study was to evaluate the *in vitro* activity of fosfomycin under different physiological concentrations of inorganic phosphate (Pi).

Methods: The wild-type BW25113 strain, four isogenic mutants (ΔglpT, ΔuhpT, ΔglpT-uhpT, and ΔphoB) and six clinical isolates of *Escherichia coli* with different fosfomycin susceptibilities were used. EUCAST breakpoints were used. Susceptibility was evaluated by agar dilution using standard Mueller–Hinton agar (Pi concentration of 1 mM similar to human plasma concentration) and supplemented with Pi (13 and 42 mM, minimum and maximum urinary Pi concentrations) and/or glucose-6-phosphate (25 mg/L). Fosfomycin transporter promoter activity was assayed using P

Results: All the strains showed decreased susceptibility to fosfomycin linked to increased Pi concentrations (1–4 log₂ dilution differences from 1 to 13 mM, and 1–8 log₂ dilution differences at 42 mM Pi). Changes in phosphate concentration did not affect the expression of fosfomycin transporters. By increasing Pi concentrations higher fosfomycin EC₅₀ bacterial viability was observed, except against ΔglpT-uhpT. The increase in Pi reduced the bactericidal effect of fosfomycin.

Discussion: Pi variations in physiological fluids may reduce fosfomycin activity against *E. coli*. Elevated Pi concentrations in urine may explain oral fosfomycin failure in non-wild-type but fosfomycin-susceptible *E. coli* strains.

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**Introduction**

Fosfomycin is a bactericidal broad-spectrum antimicrobial, approved for the treatment of uncomplicated urinary tract infections, soft-tissue infections and sepsis caused by Enterobacteriales [1]. Fosfomycin uptake is mediated by the uptake systems for sn-glycerol-3-phosphate (GlpT) and hexose-6-phosphates (UhpT) via the counterflow of inorganic phosphate...
(Pi) from the cell [2]. This intracellular Pi is maintained within the range 1–10 mM for *Escherichia coli* [3] by Pi importers (PitA, PitB and PitSCAB) and exporters (PitA, PitB, GlpT, UhpT), and controlled by the histidine kinase PhoR and the response regulator PhoB [3]. Human Pi levels vary among the different tissues and fluids, being maintained within a narrow range through a complex interplay between intestinal absorption, exchange with intracellular and bone storage pools, and renal tubular reabsorption [4].

Thus, the aim of the present study was to evaluate the *in vitro* activity of fosfomycin mimicking Pi physiological concentrations against a collection of isogenic *E. coli* mutants and clinical isolates.

**Material and methods**

**Bacterial strains and plasmids**

The wild-type *E. coli* BW25113 strain and isogenic mutants (Δ*glpT*, Δ*uhpT* and Δ*phoB*) from the KEIO collection and the double mutant Δ*glpT-uhpT*, generated by phage P1vir transduction [5], were used. Six urinary clinical *E. coli* isolates (Ec20190430, Ec20190556, Ec20190736, Ec2019800, Ec20200150 and Ec20200178) with different fosfomycin resistance from the Andalusian reference laboratory for molecular typing of nosocomial pathogens (PIRASOA programme) were included. Bacterial whole genome sequencing was performed as described in the supplementary material. *Escherichia coli* ATCC 25922 was used as control strain for the susceptibility tests. pUA66-Δ*glpT::gfp*mut2 and pUA66-Δ*uhpT::gfp*mut2 promoter fusions were constructed as described by Zaslaver et al. [6].

**Bacterial growth medium**

Mueller—Hinton Broth (MHB) and Mueller—Hinton Agar (MHA) were used with different concentrations of Pi, by adjusting standard Mueller—Hinton medium (Pi = 1 mM, similar to plasma concentration (0.8–1.4 mM)) with Na2HPO4 to 13 and 42 mM Pi, corresponding to the minimum and maximum concentrations of Pi in urine, adjusted for a 1-L volume (13–42 mmol/24 hr) or 28 mM (average concentration of Pi in urine) [4].

**Susceptibility testing**

Fosfomycin MICs were determined by agar dilution [7], in standard MHA (reference) or supplemented with Pi (13 and 42 mM Pi). Pi concentrations in Mueller—Hinton were quantified as described in the supplementary material. EUCAST breakpoints were used for intravenous fosfomycin performed at 1 mM of Pi (i.e. R ≥ 64 mg/L) and for oral fosfomycin at 13 and 42 mM of Pi (i.e. R ≥ 16 mg/L) according to the Pi concentrations in blood and urine, respectively.

**Fosfomycin activity**

Fosfomycin transporter expressions were assayed by monitoring the fluorescence accumulation in *E. coli* MG1655 carrying pUA66-Δ*glpT::gfp*mut2 or pUA66-Δ*uhpT::gfp*mut2 reporters as described in supplementary data.

Bacterial growth curves were performed in triplicate using 5 × 10⁵ CFU/ml in 96-well plates with 200 µL of volume of standard MHB or supplemented with Pi (13 and 42 mM) with 25 mg/L of glucose-6-phosphate. Fosfomycin concentrations ranged from 1 to 1024 mg/L and controls without drug were used. Bacterial viability was quantified spectrophotometrically (595 nm) at 24 hr with an Infinite 200 Pro plate reader. Fosfomycin concentration that reduces bacterial viability to 50% (EC₅₀) was estimated by fitting the bacterial viability to the equation: Viability (%) = 100 × (1 + fosfomycin concentration / EC₅₀). The mean fosfomycin EC₅₀ values were compared using the ANOVA and Tukey and Bonferroni post hoc tests.

Results

**Susceptibility testing**

The fosfomycin MICs are shown in Table 1. Increasing MHA Pi concentrations from 1 to 13 mM, 1–4 log₂ dilution differences were observed. Clinical category changes occurred for Δ*glpT* and Ec20190736, Ec20200150 and Ec20200178. The susceptible strains changed to resistant using 42 mM Pi (1–8 log₂ dilution differences).

The Δ*uhpT* and Δ*glpT-uhpT* mutants, Ec20190430 (Δ*glpT-uhpB*) and Ec20190556 were resistant irrespective of the Pi concentration. The isogenic mutant Δ*phoB* and the wild-type strain showed similar MICs at all Pi concentrations tested. Similar results were observed with Δ*glpT* with a 1 log₂ and 2 log₂ increase at Pi 13 and 42 mM, respectively, with respect to the wild-type strain.

**Fosfomycin activity**

The increased Pi concentration did not cause significant changes in *glpT* or *uhpT* transcription (Fig. S1).

Fosfomycin EC₅₀ under increasing Pi concentrations are shown in Table 2 and Fig. S2. Concentration of 13 mM Pi raised the fosfomycin EC₅₀ for the wild-type (1.42 vs. 0.34 mg/L) and Δ*glpT* (1.62 vs. 0.32 mg/L) with respect to 1 mM Pi. Concentrations of 42 mM Pi also increased fosfomycin EC₅₀ with respect to 1 and 13 mM Pi, for the wild-type (8.9 vs. 0.34 and 1.42 mg/L) and Δ*uhpT* (81.28 vs. 0.32 and 1.62 mg/L) and Δ*uhpT* (65.15 vs. 11.75 and 24.79 mg/L). Pi increased from 1 to 13 mM showed a 2 log₂ increase in fosfomycin concentration

<table>
<thead>
<tr>
<th>Strains</th>
<th>Pi concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1mM (standard)</td>
</tr>
<tr>
<td>BW25113 wild type</td>
<td>4 (S)</td>
</tr>
<tr>
<td>BW25113 Δ<em>glpT</em></td>
<td>4 (S)</td>
</tr>
<tr>
<td>BW25113 Δ<em>uhpT</em></td>
<td>128 (R)</td>
</tr>
<tr>
<td>BW25113 Δ<em>glpT-uhpT</em></td>
<td>512 (R)</td>
</tr>
<tr>
<td>BW25113 Δ<em>uhpB</em></td>
<td>4 (S)</td>
</tr>
<tr>
<td>Ec20190430</td>
<td>512 (R)</td>
</tr>
<tr>
<td>Ec20190556</td>
<td>64 (R)</td>
</tr>
<tr>
<td>Ec20190736</td>
<td>1 (S)</td>
</tr>
<tr>
<td>Ec2019800</td>
<td>2 (S)</td>
</tr>
<tr>
<td>Ec20200150</td>
<td>8 (S)</td>
</tr>
<tr>
<td>Ec20200178</td>
<td>4 (S)</td>
</tr>
</tbody>
</table>

Clinical categories for intravenous fosfomycin at 1 mM of Pi (R ≥ 64 mg/L) and oral fosfomycin at Pi 13 and 42 mM (R ≥ 16mg/L) are shown as susceptible (S) or resistant (R). Bold indicates changes in the clinical category.
able to inhibit bacterial viability, a 4 log₂ dilution increase at 42 mM Pi in the wild-type and ΔuhpT strains, and 8 log₂ increase in the ΔglpT strain. None of these changes were observed for ΔglpT-uhpT. The time—kill assay results (described in the supplementary material) showed that all fosfomycin concentrations exhibited bactericidal activity (>3 log CFU/mL decrease) against the wild-type strain, but fosfomycin activity was more affected in mutants, showing bacteriostatic activity or even bacterial regrowth at higher Pi concentrations.

### Table 2
Summary of EC₅₀ values calculated from fosfomycin dose—response experiments generated by measuring bacterial viability over 24 hr at different Pi concentrations

<table>
<thead>
<tr>
<th>Pi concentration</th>
<th>Wild type</th>
<th>ΔglpT</th>
<th>ΔuhpT</th>
<th>ΔglpT-uhpT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM</td>
<td>0.34 (0.26, 0.42)</td>
<td>0.32 (0.18, 0.48)</td>
<td>11.75 (6.7, 20.74)</td>
<td>260.4 (188.9, 360.5)</td>
</tr>
<tr>
<td>13 mM</td>
<td>1.42 (1.1, 1.82)</td>
<td>1.62 (1.15, 2.25)</td>
<td>24.79 (16.82, 36.41)</td>
<td>334.4 (240.2, 469.3)</td>
</tr>
<tr>
<td>42 mM</td>
<td>8.9⁹ (6.24, 12.56)</td>
<td>81.28⁵ (57.63, 114.1)</td>
<td>65.1⁵ (45.3, 92.62)</td>
<td>367.1 (248.4, 549.5)</td>
</tr>
</tbody>
</table>

a p < 0.05 with respect to 1 mM Pi,
b p < 0.05 with respect to 13 mM Pi.

### Discussion

The present study showed a reduction of fosfomycin in vitro activity as the Pi concentration increased, focusing on Pi and fosfomycin blood and urine concentrations where should exert its antimicrobial activity. This phenomenon was observed in susceptibility testing, viability and time—kill assays in both the isogenic and clinical isolate collections.

Environmental variables and the presence of different carbon sources have a great impact on fosfomycin activity [10–13], and the importance of phosphate homeostasis in fosfomycin activity was recently highlighted by Turner et al., showing a strong selection of fosfomycin mutants for the phosphate uptake and catabolism operon and the phosphate transporter [14].

In a previous study aimed at understanding how urinary tract infections affected fosfomycin activity against E. coli strains harbouring chromosomal mutations involved in fosfomycin uptake, urine at pH 7 reduced fosfomycin activity, especially against the null mutants ΔglpT and ΔptsI [12]. Among other factors, the different concentrations of Pi could explain, in part, these observations.

Recently, a dynamic bladder infection model simulating oral therapy using MHB, and human and synthetic urine, showed a lack of correlation between fosfomycin MICs determined using agar dilution or broth microdilution with MHB supplemented with glucose-6-phosphate and fosfomycin activity [15], underlining the gap between fosfomycin susceptibility tests and outcome possibly by the relationship between bacterial metabolism and fosfomycin activity.

The main limitation of our in vitro study is that Mueller–Hinton composition may have conditioned fosfomycin activity due to the lack of other components present in blood or urine. Also, the limited number of clinical strains with specific genotypes may have biased the conclusions. In conclusion, Pi content modifies fosfomycin activity at concentrations achieved using standard oral or parenteral dosages. Furthermore, Mueller–Hinton media are suitable for fosfomycin susceptibility testing due to their low Pi content, but may overestimate in vitro activity with respect to infection sites with higher Pi levels. Both observations should be validated with a larger number of clinical isolates, together with their clinical outcomes especially in cases of Pi homeostasis disorders, to establish the clinical significance.

### Transparency declaration

This study has been funded by Instituto de Salud Carlos III through the projects P116/01824, P119/01645 and REIPI RD16/0016/0001 (co-funded by European Regional Development Fund “A way to make Europe”), Miriam Ortiz-Padilla is supported by a PFIS grant from the Instituto de Salud Carlos III (FI17/00235). Inés Portillo-Calderón is supported by a Rio Hortega grant from the Instituto de Salud Carlos III (CM20/0092). (Co-funded by European Social Fund “Investing in your future”). The funders had no role in the design, collection of data, analysis and writing of the manuscript or the decision to publish. This study was presented in part at ECCMID 2021 (Abstract number: 00868).

### Author contributions

F.D.P., J.M.R.M. and A.P.H. conceived the idea and experimental design. M.O.P., I.P.C. and B.G.I. conducted the phenotypic experiments. M.O.P., I.P.C. N.M.A. and B.G.I. conducted the molecular experiments. V.M.B. quantified Pi concentrations in Mueller–Hinton. F.D.P. and A.P.H. supervised the project. M.O.P., I.P.C., J.M.R.M., J.R.B. and F.D.P. performed the analysis and interpreted the results. F.D.P., A.P.H. and M.O.P. wrote the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2021.09.037.

### References


[5] Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2006;2. 2006.0001 (co-funded by European Regional Development Fund “Investing in your future”). Miriam Ortiz-Padilla is supported by a PFIS grant from the Instituto de Salud Carlos III (FI17/00235). Inés Portillo-Calderón is supported by a Rio Hortega grant from the Instituto de Salud Carlos III (CM20/0092). (Co-funded by European Social Fund “Investing in your future”). The funders had no role in the design, collection of data, analysis and writing of the manuscript or the decision to publish. This study was presented in part at ECCMID 2021 (Abstract number: 00868).


