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Amikacin Combined with Fosfomycin for Treatment of Neonatal Sepsis in the Setting of Highly Prevalent Antimicrobial Resistance

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21 Running head: Amikacin and fosfomycin combination pharmacodynamics

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ABSTRACT

Antimicrobial resistance (particularly by extended spectrum β-lactamase and aminoglycoside modifying enzyme production) in neonatal sepsis is a global problem, particularly in low- and middle-income countries, causing significant mortality. High rates of resistance are reported for the current WHO-recommended first-line antibiotic regimen for neonatal sepsis; ampicillin and gentamicin. We assessed the utility of fosfomycin and amikacin as a potential alternative regimen to be used in settings of increasingly prevalent antimicrobial resistance.

The combination was studied in a 16 arm dose ranged hollow-fiber infection model (HFIM) experiment. The combination of amikacin and fosfomycin enhanced bactericidal activity and prevented emergence of resistance compared to monotherapy of either antibiotic. Modelling of the experimental quantitative outputs and data from checkerboard assays, indicated synergy.

We further assessed the combination regimen at clinically relevant doses in HFIM with nine Enterobacterales strains with high fosfomycin/amikacin MICs and demonstrated successful kill to sterilisation in 6/9 strains. From these data, we propose a novel combination breakpoint threshold for microbiological success for this antimicrobial combination against Enterobacterales - MIC_F * MIC_A < 256 (where MIC_F and MIC_A are MICs for fosfomycin and amikacin). Monte Carlo simulations predict that a standard fosfomycin/amikacin neonatal regimen will achieve a >99% probability of pharmacodynamic success for strains with MICs below this threshold.

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We conclude that the combination of fosfomycin with amikacin is a viable regimen for the empiric treatment of neonatal sepsis and is suitable for further clinical assessment in a randomised controlled trial.

Introduction

Neonatal sepsis is a common condition with a high mortality (1). Leading causative pathogens are both Gram-negative (e.g. *E. coli*, *K. pneumoniae*) and Gram-positive organisms (e.g. *Staphylococcus aureus, Streptococcus agalactiae* (Group B streptococci - GBS)) (1). Neonatal sepsis accounts for an estimated 430,000 - 680,000 deaths annually, with the highest mortality in low- and middle-income countries (LMICs) (2, 3). The World Health Organisation (WHO) currently recommends a narrow-spectrum β-lactam agent (e.g. amoxicillin or penicillin G) in combination with gentamicin as the first line empiric regimen to treat neonatal sepsis (4, 5). This regimen has an acceptable safety profile, is active against common causative wild-type organisms, is inexpensive and feasible to administer. However, clinical efficacy is increasingly compromised by the rise of antimicrobial resistance (AMR).

Multiple epidemiological studies of neonatal sepsis demonstrate significant levels of drug resistance, particularly to β-lactams and gentamicin (6–12), with a variety of increasingly prevalent resistance mechanisms such as extended spectrum β-lactamases (ESBLs) and aminoglycoside modifying enzymes (AMEs). In hospital settings, resistance rates of Gram-negative bacteria causing neonatal sepsis to amoxicillin and gentamicin are approximately 80% and 60%, respectively, with some regional variation (6–12). Alternative options are urgently required for the treatment of neonatal sepsis caused by multi- and extremely-drug resistant (MDR and XDR) bacteria and suitable for use in LMIC settings.

A potential replacement regimen would need to provide spectrum of activity against the commonly encountered pathogens and resistance motifs. Additionally, if the regimen were a

combination of two agents, a favourable pharmacodynamic interaction would benefical. Antimicrobial interactions can be defined by several metrics and definitions (13). However, the interaction model described by Greco based on Loewe additivity (14, 15) allows determination and quantification of any interaction with precision and without arbitrary thresholds for determining the natures of interaction.

Amikacin and fosfomycin have several attributes that make them potential candidates for use in neonatal sepsis. They are off-patent with a neonatal licence, have an acceptable safety profile with limited toxicities (16, 17), and have efficacy against commonly encountered multidrug resistant (MDR) pathogens. We therefore studied the potential utility of this combination for neonatal sepsis by assessing *in vitro* activity, the nature and extent of any pharmacodynamic interaction using checkerboard assays and hollow fiber infection models (HFIMs), and defined candidate combination regimens suitable for further clinical study.

Results

In vitro susceptibility testing

A panel of 40 strains of bacterial species was assembled to give a representative range of bacteria that cause neonatal sepsis in a LMIC setting, with a majority of strains harbouring relevant resistance motifs for geographic regions of interest. These include 10 methicillin-resistant *Staphyloccocus aureus* (MRSA) strains, 10 *E. coli* and 10 *K. pneumoniae* strains (all ESBL or carbapenemase producers), and 10 wild-type *S. agalactiae* strains (Table S1). The MIC distributions for fosfomycin and amikacin against this panel of strains are shown in Table S2. The modal amikacin MIC was 2-4 mg/L (excluding the intrinsically resistant *S. agalactiae*, inhibited by a modal MIC of >32 mg/L); the modal fosfomycin MIC was 2 mg/L (excluding the *K. pneumoniae* strains, which have a modal MIC of >32mg/L, likely due to a high incidence of chromosomal FosA (18)).

In vitro drug-drug interaction modelling

Checkerboard assays were performed on a selection of the neonatal sepsis panel strains (n=16). These strains were selected on the basis of having MICs >0.0625mg/L and <32mg/L for fosfomycin and amikacin. An interaction model originally developed by Greco (14) was fitted to the dataset to estimate a pharmacodynamic interaction parameter, α, for each strain (Fig. 1). A value of α for the interaction of two agents is interpreted as follows: a lower bound of the 95% CI of $\alpha > 0$ indicates a synergistic interaction; an upper bound of the 95% CI of $\alpha < 0$ indicates an antagonistic interaction; a 95% CI crossing 0 indicates no evidence of interaction i.e. simple

101 additivity (14)). A total of 9/16 individual strains had CIs >0 (and therefore indicated synergy);
102 the remaining 7/16 strains had CIs crossing 0 (and therefore demonstrated no evidence of
103 interaction). When the α value output of the models fitted to each strains were combined in a
104 meta-analysis, the combined α interaction value was 0.1705 (95% CI 0.0811 to 0.2599), with
105 low inter-strain heterogeneity (I^2 = 30.7%, p value = 0.383) indicating a synergistic effect
106 observed across all species/strains tested.

107

108 **Pharmacodynamic interaction of fosfomycin and amikacin using neonatal PK**

109 To determine the nature and magnitude of the pharmacodynamic interaction between
110 fosfomycin and amikacin using neonatal concentration-time profiles, a hollow fiber infection
111 model (HFIM) was used (Fig. S1) using the *E coli* ST195 strain, a CTX-M-14 producer from Laos
112 (amikacin MIC 4 mg/L; fosfomycin MIC 1 mg/L) (19) . These experiments were conducted
113 following preliminary dose-finding experiments with each drug alone to define informative
114 parts of the drug exposure-response and drug exposure-emergence of resistance relationships.
115 For fosfomycin, the EC_{20}, EC_{50}, and EC_{80} for bactericidal effect were achieved with $fAUC_{0\text{-}24}$ of
116 25, 200 and 400 mg*h/L, respectively. For amikacin, the EC_{20}, EC_{50}, and EC_{80} were achieved with
117 $fAUC_{0\text{-}24}$ of 50, 200 and 380 mg*h/L, respectively.

118 The pharmacodynamics of the fosfomycin-amikacin combination was determined in a 16-arm
119 4x4 experiment that included no-treatment controls, each drug alone at the three doses, and
120 an interaction matrix of all 2-drug dose combinations as shown in Fig. 2. When administered
121 alone, increasing fosfomycin exposures resulted in profound early bacterial killing. However,

failure to achieve sterility led to rapid regrowth, with emergence of a resistant clone(s) with fosfomycin MICs of ≥128mg/L, with maximal emergent resistance at *f*AUC$_{0\text{-}24}$ of 50 and 200 mg*h/L (Fig. 2, Panels 1-4). Similarly, progressively increasing exposures of amikacin as monotherapy led to initial suppression of logarithmic growth with subsequent exposure-dependent emergence of a resistant subpopulation with amikacin MICs ≥16mg/L, with maximal emergent resistance at *f*AUC$_{0\text{-}24}$ of 380 mg*h/L (Fig. 2, Panels 1,5, 9, & 13).

In combination, fosfomycin and amikacin achieved a greater magnitude of initial bacterial kill, with delayed and reduced emergence of resistance to fosfomycin and amikacin, compared with equivalent drug exposures in monotherapy. Higher combination exposures achieved sterility. The relationship between drug exposure and the emergence of resistance with each drug administered alone formed an 'inverted U' (20). Fosfomycin and amikacin in combination resulted in the suppression of resistance that occured at comparable drug exposures in monotherapy of each drug (Fig. 2, Panels 11,12 & 14-16). As the exposure of the other antibiotic increased, the 'inverted U' shifted to the left as emergence of resistance was progressively suppressed (Fig. 3).

The nature and magnitude of the pharmacodynamic interaction between fosfomycin and amikacin was estimated by fitting a pharmacodynamic interaction model to the PK-PD data (Table 1). The R-squared values for the observered vs individual predicted values were 0.875 (free fosfomycin concentrations), 0.963 (free amikacin concentrations), 0.869 (total bacterial count), 0.944 (fosfomycin-resistant bacterial count) and 0.669 (amikacin-resistant bacterial count). There were synergistic relationships for the effects of the combination on susceptible, fosfomycin-resistant, and amikacin-resistant bacteria with α values of 13.046 [95% CI 0.761 –

25.331], 20.520 [95% CI 11.727 – 29.313], and 25.227 [95% CI 14.485 – 35.969], respectively. Hence, the combination of fosfomycin and amikacin was synergistic in terms of killing both drug-susceptible and -resistant subpopulations.

Assessment of a Neonatal Combination Regimen of Fosfomycin and Amikacin

We assessed the pharmacodynamics of the combination of fosfomycin and amikacin using neonatal concentration-time profiles of each drug over a 7 day period. For amikacin, we used a standard neonatal dose of 15 mg/kg q24h (21) and a median neonatal half-life of 7 hr (22). For fosfomycin we used a neonatal dose of 100mg/kg q12h with a half-life of 5.2 hr, based on preliminary data from the NeoFosfo trial (23). We selected nine Gram-negative bacteria as the challenge strains that had a range of MICs to both drugs and had different mechanisms of resistance (Table 2). We successfully recapitulated the target free drug PK profiles associated with each regimen (data not shown).

The summary pharmacodynamics are shown in Fig. 4 (full pharmacodynamic output are shown in Fig. S2-10). When administered alone, amikacin and fosfomycin failed to achieve extinction in 9/9 and 7/9 strains, respectively. All arms with strains inhibited by fosfomycin MICs >4mg/L treated with fosfomycin monotherapy had rapid emergence of resistance within 24h. The three strains inhibited by fosfomycin MICs ≤4mg/L were either killed to sterility (two strains) or had delayed emergence of resistance towards the end of the experiment. In contrast, the combination regimen achieved extinction in 6/9 strains. The strains for which the combination failed were all inhibited by MICs ≥ 32mg/L and ≥ 8mg/L for fosfomycin and amikacin,

165 respectively. The distribution of combined fosfomycin and amikacin MICs versus response is
166 shown in Fig. 4a. In this figure, a plane (or line) delineated two groups of strains, defined by the
167 fosfomycin/amikacin MICs, that predicted success (defined as sterility at the end of the
168 experiment) and failure. This 'breakpoint plane' was described in the following Cartesian format
169 $MIC_A * MIC_F = 256$, where MIC_A and MIC_F are amikacin and fosfomycin MICs, respectively. In
170 a clinical context, this means that if the product of the amikacin and fosfomycin MICs inhibiting
171 a bacterial pathogen is < 256, then treatment with a neonatal regimen of fosfomycin and
172 amikacin in combination can be predicted to succeed (i.e. the bacterium is 'sensitive' to this
173 combination).

174 The amikacin/fosfomycin combination success data can also be arranged according to the
175 *f*AUC:MIC ratio for each drug, as shown in Fig. 4b, with a similar plane describing the threshold
176 for successful treatment with the combination. This target plane can be described with the
177 form $(fAUC_F/\ MIC_F) * (fAUC_A/\ MIC_A)\ = 2709.5$ (where F and A subscripts denote
178 fosfomycin and amikacin *f*AUCs and MICs respectively). Interpreted in a clinical context, if the
179 product of the amikacin and fosfomycin *f*AUC:MIC ratios is >2709.5, then the target for
180 pharmacodynamic success has been met, with predicted treatment success.

181

182 **Monte Carlo Simulations**

183 Amikacin and fosfomycin *f*AUCs for 10,000 neonates were created using a Monte Carlo
184 simulation from a neonatal fosfomycin model that included neonatal covariate distributions
185 based on a neonatal cohorts from the NeoFosfo trial and a recently completed global neonatal

sepsis observational study (NeoOBS) (23, 24) and a recently published neonatal amikacin model (25). Simulated dosing regimes were fosfomycin 100mg/kg q12 for neonates ≤7 days old and 150mg/kg q12 for neonates >7 days, as suggested by the NeoFosfo trial results and the EMA dosing recommendations (23, 26). Simulated amikacin dosages were 15mg/kg q24 for all neonates > 2kg; neonates weighing ≤ 2kg were dosed at q48 if ≤7 days old and q36 if >7 days old (27).

Using the target relationships defined above, we calculated a combined probability of pharmacodyamic target attainment for both drugs across MIC ranges (1 – 256 mg/L) (Table 3). These simulated *f*AUCs demonstrated ≥99% predicted target attainment for Enterobacterales with amikacin and fosfomycin MICs below the 'breakpoint plane'. This indicates a high likelihood that fosfomycin and amikacin in combination at the simulated dosing regimens (i.e. at standard neonatal doses) will successfully treat neonatal sepsis caused by these pathogens.

198 **Discussion**

199 In both static and dynamic *in vitro* pharmacological models there was unequivocal synergistic
200 interactions between amikacin and fosfomycin when measuring by bactericidal killing and the
201 prevention of emergence of antimicrobial resistance. In particular, the addition of increasing
202 doses of the second agents suppresses the 'inverted U' of antimicrobial resistance emergence
203 (20) (Fig. 3) preventing the resistance observed at equivalent doses in monotherapy. These
204 characteristics are unaffected by the presence of resistance mechanisms that render first line
205 agents ineffective (e.g. ESBL and AMEs) in the bacteria tested in our experiments. The
206 combination fosfomycin and amikacin is therefore a potentially useful regiment for empiric
207 treatment of neonatal sepsis in the context of high prevalence of these resistance mechanisms

208 Prediction of antimicrobial success has traditionally been conceived using breakpoint
209 thresholds on a scale of a single drug concentration, with the treatment success dependent
210 upon the bacteria being inhibited by a MIC being above or below a certain threshold on this
211 scale. Our data suggests that using conventional monotherapy breakpoints is of limited value in
212 combination antibiotics (Fig. 4). Here, we propose a novel two-dimensional breakpoint
213 concentration threshold for treatment success defined by the Cartesian function of the
214 pathogen's fosfomycin and amikacin MIC; $MIC_A * MIC_F = 256$, where A and F subscripts
215 denote amikacin and fosfomycin MICs respectively. Enterobacterales pathogens that are
216 inhibited by amikacin and fosfomycin MICs lying beneath this threshold (i.e. $MIC_A * MIC_F < 256$)
217 can be predicted to be successfully treated by the standard regimen of these agents used in
218 neonates i.e. it is specific to a neonatal context.

In a further extension, we also propose a novel combination pharmacodynamic target threshold for the combination regimen for predicted treatment success, described in the following Cartersian format: $(fAUC_F/\ MIC_F) * (fAUC_A/\ MIC_A)\ = 2709.5$. The probabilities of standard neonatal regimens of these drugs attaining this threshold, for bacteria inhibited by a range of MIC combinations and incorporating the variability of neonatal drug exposure, are summarised in Table 3.

We aimed to ensure a diversity of resistance mechanisms across the strains used, with commonly encountered resistance motifs in LMICs represented, acknowledging we are limited to the nine strains used. Whilst it is possible that bacteria with resistance mechanisms not examined in our experiments do not follow the relationship described, the MIC provides an integrative measure of potency regardless of the molecular mechanism of resistance, and can be used to predict pharmacodynamic response, as with conventional breakpoints.

In our HFIM experiments the monotherapy arms failed with strains inhibited by fosfomycin and amikacin MICs below their EUCAST breakpoint concentrations (32mg/L for fosfomycin and 8mg/L for amikacin (28)). The underperformance of amikacin partially supports the recent downward revision of aminoglycoside breakpoint concentrations by EUCAST with a recommendation to avoid aminoglycoside monotherapy for systemic infections (28), but also reflects the observed greater tendency of aminoglycoside exposure to generate emergence of resistant small-colony variants *in vitro* than is observed *in vivo* (29). Failure of fosfomycin as monotherapy for strains inhibited by MICs >4mg/L supports suggestions that the breakpoint concentration for neonatal systemic infections should be lower than the currently stated EUCAST breakpoint for adult systemic infections of 32mg/L (28) (as has previously also been

241 suggested in an adult context too (30)). However, the ideal breakpoint concentration for
242 fosfomycin alone is difficult to define because this agent should not be used as monotherapy
243 due to potential for rapid emergence of resistance (31, 32).

244 There is an increasing number of experimental models of neonatal infection and sepsis (33, 34).
245 HFIMs has been previously used to explore the pharmacodynamics of vancomycin and
246 teicoplanin for neonatal sepsis (33, 35). HFIM has the advantage of enabling the simulation of
247 neonatal pharmacokinetics to explore drug exposure effect and drug exposure resistance
248 relationships that are specific to this special population. This is extremely difficult to achieve in
249 laboratory animal models, due to inherent pharmacokinetic differences with humans.
250 Furthermore, laboratory animal models of bacteraemia have additional difficulties in
251 establishing pharmacodynamic relationships to due to the relatively low and intermittently
252 detectable bacterial densities. The HFIM overcomes these limitations.

253 However, the HFIM does not replicate the anatomical barriers that may be important for
254 infections of the lung and brain, and does not contain any immunological effectors (even if
255 these are immature in neonates) that may contribute to antimicrobial activity. Furthermore,
256 the relatively high density of the inoculum used in HFIM to ensure reproducible results (circa.
257 10^6 cfu/mL) is higher than the estimates for the bacterial density in the bloodstream of
258 neonates with sepsis (circa. 10^0-10^3 CFU/mL) (36, 37). For these reasons, the conclusions from
259 the HFIM may be conservative and represent a worst-case scenario for regimen identification.

260 Furthermore, the conclusions of these experiments are applicable only to the treatment of
261 systemic infections (i.e. neonatal sepsis) given the replication of neonatal systemic drug
262 exposures. Whilst both amikacin and fosfomycin have a degree of CSF penetration (amikacin

has a CSF partition coefficient of 0.1 in neonates (38); fosfomycin has a CSF coefficient of 0.15-0.2 in adults (39), with neonatal data expected in the Neofosfo trial (23)), the CSF drug exposures and the behaviour of bacterial inoculums in neonatal meningitis will be different to those modelled in this system.

Despite these limitations, we conclude these experiments demonstrate that the regimen of fosfomycin and amikacin in combination is synergistic in both bactericidal effect and prevention of acquired antimicrobial resistance to either drug, with a defined threshold for probable treatment success. Additionally both agents have attributes that make them suitable for use in LMIC settings: i) Stability at room temperature (40, 41); ii) Ease of administration with once or twice daily dosing; iii) Minimal toxicities; iv) Off-patent status, and therefore potential affordability; v) Potential activity, in combination, to the predominant bacterial causes of neonatal sepsis. We conclude that this combination regimen could be considered appropriate for empiric treatment of neonatal sepsis in LMIC settings.

276 **Methods and Materials**

277 ***Antimicrobial agents.*** Amikacin (Alfa Aesar, Haverhill), and fosfomycin (Sigma-Aldrich, St Louis)
278 were purchased. Both agents were stored at 2-8^{o}C in anhydrous form. Fresh solutions were
279 prepared in sterile distilled water prior to any use. For the *in vitro* hollow fiber infection model
280 (HFIM) experiments, a licensed pharamaceutical preparation of fosfomycin (Fomicyt, Kent
281 Pharmaceuticals Ltd) were used and were prepared using sterile distilled water.

282 ***Media and agar***. Cation-adjusted Muller Hinton broth (MHB) (Sigma-Aldrich, St Louis) was used
283 as the primary media in all experiments. As fosfomycin requires the presence of glucose-6-
284 phosphate (G6P) for bacterial cell entry (42) the MHB was supplemented with 25mg/L G6P
285 (Sigma-Aldrich, St Louis) in experiments where fosfomycin is used. Mueller Hinton agar (MHA)
286 was used in all agar plates. Commercially pre-prepared 20mL round MHA plates (Fisher
287 Scientific, Waltham) or self-prepared 50ml square MHA plates (MHA from Sigma-Aldrich;
288 square plates from VWR, Radnor) were used in all experiments. For drug-containing plates,
289 MHA was supplemented with antibiotic (with 25mg/L G6P in the case of fosfomycin) and
290 prepared within each antibiotic's stability limits. Drug concentrations in agar were four times
291 the MIC of the specific bacterial strain used in a given experiment.

292 ***Bacterial Isolates*.** Isolates were supplied by JMI, IHMA, Public Health England (PHE), LGC
293 standards, University of Birmingham, University of Oxford, and Royal Liverpool University
294 Hospital. For the initial non-dynamic *in vitro* experiments, a collection of strains was collated
295 representing a range of common possible neonatal sepsis bacterial pathogens and resistance
296 mechanisms in an AMR prevalent environment. In total, this included 10 strains of each of the
297 following: Group B streptococci, methicillin resistant *Staphylococcus aureus* (MRSA), *Escherichia*

298 *coli,* and *Klebsiella pneumoniae*. All of the Gram-negative bacteria were extended spectrum β-
299 lactamase (ESBL) (nine *E. coli* and nine *K. pneumoniae* strains*)* or carbapenemase producers
300 (one *E. coli* and one *K. pneumoniae strain)*. Some of these strains were used in the HFIM based
301 on their MICs, including a further two *K. pneumoniae* and one *E. coli* (ESBL producers) not
302 included in the original 40 strain panel (full details of the isolates are detailed in Table S1). All
303 isolates were stored in glycerol at -80°C and sub-cultured onto two MHA plates for 18-24h at
304 37°C prior to each experiment. In each non-HFIM experiment, colonies were suspended in PBS
305 to MacFarland standard 0.5 ($1x10^8$ CFU/mL) and diluted to the target concentration. For HFIM
306 experiments, bacteria was incubated in MHB until the bacteria entered exponential growth,
307 and quantified by optical density (600nm) according to a strain specific standard growth curve.

308 ***Antimicrobial susceptibility testing*.** Fosfomycin and amikacin minimum inhibitory
309 concentrations (MICs) for the panel of representative neonatal sepsis bacterial pathogens were
310 determined using the EUCAST broth microdilution methodology (43). *E. coli* ATCC 25922 or *S.*
311 *aureus* ATCC 29213 were used as controls in all experiments. The antibiotic gradient strip assay
312 method was used for isolates from the hollow fiber experiment. Briefly, an inoculum of the
313 isolate was made using a suspension of a sweep of colonies into PBS to a McFarland standard of
314 0.5. A lawn of the inoculum was plated onto a MHA plate and an antibiotic gradient strip (Etest,
315 Biomerieux, Marcy-l'Étoile, France) placed on the plate, which is subsequently incubated for 18-
316 24h at 37°C before reading. Interpretation of susceptibility was determined using 2020 EUCAST
317 breakpoints (28). The breakpoint for IV fosfomycin was used for fosfomycin MIC interpretation.

318 ***In vitro pharmacodynamic assays*.** Checkerboard assays were used on selected strains to assess
319 the pharmacodynamic interaction of the fosfomycin/amikacin combination. Strains were

selected based on having MICs ≤32mg/L and >0.0625 mg/L to both fosfomycin and amikacin. 100 μL of antimicrobials in sterile distilled water were added to the an 8x8 grid on a 96 well plate, with concentration gradients created with 1:2 serial dilutions along each axis, with the final row/column having 0 mg/L of the appropriate drug. The drug concentration range used on each plate was chosen according to the drug MICs of each strain, with the maximum concentration of each antimicrobial being 4x MIC for that strain. The inoculum was made up to $1x10^6$ CFU/mL in MHB and quantified using 1:10 serial dilution onto MHA plates. 100μl of the inoculum was added to each well of the prepared checkerboard. The well containing 0 mg/mL of each drug acted as the positive control; an additional row of blank MHB on the plate acted as negative control. Plates were incubated 18-24h at 37°C before being read by optical densitometer (Varioskan, Thermo Fisher) at 600nm. Plates were considered valid if the MIC on the monotherapy rows of the checkerboard were within 1 dilution of previously determined MICs, the negative controls had no growth, and the prepared inoculum was within 6-14 x 10^5 CFU/mL.

Raw optical densitometer (OD) readings were normalised to that of the positive control. The readouts were then modelled using Greco's model of drug synergy (15) using ADAPT 5 (44), with determination of α, with confidence intervals calculated using standard error of the model outputs. Meta-analysis was performed on the output of the combination using the R package 'Metafor' (45).

Hollow Fiber Infection Model. The hollow fiber infection model (HFIM) is a well-established dynamic model stimulating the pharmacodynamic effect of antimicrobials with physiological dynamic concentrations (46). The HFIM method was used largely as described previously (33).

342 Briefly, each arm in the HFIM is set up as demonstrated in Fig. S1; monotherapy arms omit the
343 supplementary compartments. MHB is pumped into the central compartment at a rate set to
344 simulate a physiological clearance rate for the drug, with all media in the central compartment
345 above 300 mL removed via an elimination pump. The target simulated half-lives for fosfomycin
346 and amikacin were 5.1 and 7 hours respectively. The neonatal half-life of fosfomycin was
347 determined from then unpublished data from the NeoFosfo trial (23). The neonatal half-life of
348 amikacin was sourced from the SPC (47) and confirmed with other published neonatal clinical
349 PK data (48–52) To account for the difference in clearance between fosfomycin and amikacin,
350 supplementary compartments were set up according the principles laid out by Blaser (53).

351 Throughout the HFIM experiments, inoculum concentrations were determined by serial dilution
352 1:10. A total of 10μL of each dilution was pipetted onto MHA plates; one drug-free and two
353 containing either fosfomycin or amikacin. An additional 100μL of the original inoculum was
354 plated onto a drug-free MHA plate to lower the limit of detection for total bacterial
355 quantification (i.e. to 10 CFU/mL). Plates were then incubated at 37°C for 18-24 hr for drug free
356 plates, and 42-48 hr for drug-containing plates. After incubation, colonies were counted for at
357 least two dilutions and the CFU/mL of the original inoculum was calculated.

358 Preliminary monotherapy experiments were performed with the ESBL-producing ST195 *E. coli*
359 strain (fosfomycin MIC 1mg/L, amikacin MIC 4 mg/L; supplied by the University of Birmingham)
360 (19). PK and PD outputs of these experiments were modelled using Pmetrics (54) and
361 parameters simulated using ADAPT (44) to determine the fosfomycin and amikacin doses
362 required to achieve EC_{20}, EC_{50} and EC_{80} in terms of bactericidal effect within the HFIM. A 16-arm
363 HFIM experiment was performed using a 4x4 dosing matrix using these three doses and no

dose for both antibiotics in combination. The experiment was run over 96 hours, with a target initial inoculum of $1x10^6$ CFU/mL of ST195 inoculated into the hollow fiber cartridges. A dose of fosfomycin corresponding to the EC_{20}, EC_{50} and EC_{80} was administered every 12 hours to the primary central compartment only; an amikacin dose achieving the EC_{20}, EC_{50} and EC_{80} was administered to the primary and supplementary central compartments every 24 hours.

PK samples were taken for bioanalysis at four timepoints in dosing windows in days 1 and 3 of the experiment. Samples of inoculum were taken from each hollow fiber cartridge at 4 timepoints during the first 24h, then once daily before administration of dose until the 96h timepoint. Each sample was prepared and plated onto drug-free square agar plates and fosfomycin- and amikacin- containing plates, as described above. MICs from any viable colonies from each arm on the final timepoint were determined via antibiotic gradient strip assay .

Further HFIM experiments were performed assessing the effect of clinically relevant fosfomycin and amikacin doses leading to neonatal-like pharmacokinetic profile alone and in combination against a variety of bacteria with different fosfomycin and amikacin MICs. PK profiles of fosfomycin and amikacin were designed to have half-lives of 5.1 and 7 hours, with Cmax values of 250mg/L and 40mg/L respectively. These were determined from the sources used to determine the half-life, as described earlier. Nine parallel experiments were performed using nine Gram-negative strains with a wide distribution of fosfomycin and amikacin MICs (Table 2). Each individual experiment consisted of 4 arms; monotherapy arms for both fosfomycin and amikacin, a combination therapy arm, and an untreated control. As this experiment aimed to replicate clinically relevant drug exposures in neonates, each experiment lasted 7 days to reflect the typical treatment course of neonatal sepsis. Four PK samples were taken in each of

three dose intervals distributed evenly throughout the experiment. Four inoculum samples were taken on day 1, and once every 24h thereafter. These samples were quantified on drug-free, fosfomycin-, and amikacin-containing square MHA plates. MICs from any viable colonies from each arm on the final timepoint were determined via antibiotic gradient strip assay.

Amikacin Bioanalysis. The internal standard, [$^{2}H_{5}$] amikacin (Alsachim, Illkirch-Graffenstaden, France) was prepared in acetonitrile plus 5% trichloroacetic acid (TCA) (25 mg/L, Fisher Scientific, UK) and 150 µL was added to a 96-well protein precipitation plate (Phenomenex, Cheshire, UK). Fifty µL each of samples, blanks, calibrators in the range 0.5 – 50 mg/L and quality controls (0.75, 7.5 and 37.5 mg/L) were mixed with the internal standard on an orbital shaker. Liquid was drawn through the protein precipitation plate into a collection plate using a positive pressure manifold. Samples were evaporated under nitrogen (40 L/min) followed by reconstitution in water (Fisher Scientific, UK) and 0.1% heptafluorobutyric acid [Sigma-Aldrich, UK] and mixed using an orbital shaker prior to analysis by LC-MS-MS.

LC-MS-MS analysis was performed using an Agilent 1290 Infinity HPLC coupled to an Agilent 6420 triple quadrupole mass spectrometer fitted with an electrospray source controlled using Agilent MassHunter Data Acquisition software (Ver B.06.00). Analytes were injected (5 µL) onto a Discovery® HS C18 HPLC Column (2.1 mm x 50 mm, 3 µm, 50°C) and separated over a 3.5 min. gradient using a mixture of solvents A (LC-MS grade water with 0.1% (v/v) heptafluorobutyric acid) and B (HPLC grade acetonitrile with 0.1% (v/v) heptafluorobutyric acid). Separations were performed by applying a linear gradient of 2% to 98% solvent B over 3 mins at 0.5 mL/min followed by an equilibration step (0.5 mins at 2% solvent B).

407 The mass spectrometer was operated in positive ion mode using a Multiple Reaction
408 Monitoring (MRM) method with the specified mass transitions and collision energies: amikacin
409 586.4 > 163.2 (Ce 30 ev) and [$^{2}H_{5}$] amikacin 591.3 > 163.2 (Ce 30 ev). Mass spectrometry
410 readouts were processed using Agilent Mass Hunter Quantitative Analysis (Ver B.05.02).

411 Prior to sample analysis, the analytical method was validated to assess recovery and matrix
412 effects, inter- and intra-day accuracy and precision, carryover, dilution integrity, stability in
413 matrix (4 hours at room temperature and 3 freeze thaw cycles) and processed sample stability
414 (reinjection of extracts after 24hrs). The average recovery from matrix was 75.3%. The limit of
415 quantification (LLQ) was defined as 0.5 mg/L and the limit of detection (LOD) 0.25 mg/L. The
416 inter- and intra-day %CV on the three QC levels ranged from 2.5% – 5.7% and 2.9% – 6.41%
417 respectively. The analyte was found to be stable in all conditions described above.

418 **Fosfomycin Bioanalysis.** The internal standard, Ethyl Phosphonic acid (Sigma Aldrich, UK) was
419 prepared in acetonitrile (5 mg/L, Fisher Scientific UK) and 200 μL was added to a 96-well protein
420 precipitation plate (Phenomenex, Cheshire, UK). Fifty μL each of samples, blanks, calibrators in
421 the range 1 – 500 mg/L and quality controls (3.5, 35 and 350 mg/L) were mixed with the
422 internal standard on an orbital shaker. Liquid was drawn through the protein precipitation
423 plate into a collection plate using a positive pressure manifold with water and 2mM Ammonium
424 acetate (150 μL) added to each well, before sealing and mixing on an orbital shaker.

425 LC-MS-MS analysis was carried out using the same technical setup as described above.
426 Analytes were injected (5 μL) onto an Agilent ZORBAX RRHD HILIC Plus 95Å Column (2.1 mm x
427 50 mm, 1.8 μm, 40°C) and separated over a 3.5 min. gradient using a mixture of solvents A (LC-

MS grade water with 2mM (v/v) ammonium acetate) and B (HPLC grade acetonitrile). Separations were performed by applying a linear gradient of 100% to 0% solvent B over 2 mins at 0.4 mL/min followed by an equilibration step (1.5 mins at 100% solvent B).

The mass spectrometer was operated in negative ion mode using a Multiple Reaction Monitoring (MRM) method with the specified mass transitions and collision energies: fosfomycin 137.1 > 79.0 (Ce 20 ev) and EPA 109.1 > 79.0 (Ce 20 ev). Mass spectrometry readouts were processed as described above.

This fosfomycin analytical method underwent the same validation process as the amikacin method described above. The average recovery from matrix was 80.9%. The LLQ was defined as 1 mg/L and the LOD 0.5 mg/L. The inter and intra day %CV on the three QC levels ranged from 6.5% – 8.1% and 4.7% – 6.9% respectively. The analyte was found to be stable in all conditions described above.

Modelling. Population PK models were constructed using the pharmacokinetic and pharmacodynamic outputs of the hollow fiber experiments using the population PK program Pmetrics using a nonparametric adaptive grid NPAG estimation routine (54). The structural model was based on Greco's models of pharmacological synergy (15) (described in full in Text S1, Supplementary Materials).

Monte Carlo Simulation. A neonatal model for fosfomycin developed from the Neofosfo trial (23, 55) and previously published neonatal amikacin (56) was used to simulate fosfomycin/amikacin PK profiles from 10,000 neonates the linPK package in R (https://cran.r-project.org/web/packages/linpk/index.html). The simulated population was based on the

449 demographic distribution of neonates in the Neofosfo trial (23) combined with data from an
450 international multi-centre neonatal observational trial (24). From the simulated PK profiles,
451 individual *f*$AUC_{0\text{-}24h}$ values were calculated from the first 24h.

452 **Data availability:** The programs ADAPT and Pmetrics are pubically available, with instructions,
453 at https://bmsr.usc.edu/software/adapt/ and http://www.lapk.org/pmetrics.php respectively.

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464 **Declared Interest:** None

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630 **Tables**

631

Parameter	Mean	Median	**95% Credibility interval**
V1 (L)	0.459	0.469	0.416 – 0.5
V2 (L)	0.359	0.312	0.306 – 0.417
Cl1 (L/h)	0.082	0.077	0.0755 – 0.0967
Cl2 (L/h)	0.038	0.031	0.0308 – 0.0369
Kgs	1.320	1.124	1.000 - 1.579
Kks	2.698	2.922	2.700 - 3.000
$E50_1s$ (mg/L)	9.081	6.805	4.417 – 11.260
$E50_2s$ (mg/L)	11.674	6.768	4.041 – 17.540
α_s	16.288	13.046	3.439 – 29.997
Kgr1	1.375	1.324	1.239 – 1.329
Kkr1	2.384	2.221	1.933 – 2.902
$E50_1r1$ (mg/L)	34.554	28.833	28.228 – 42.833
α_{r1}	17.023	20.520	11.021 – 22.068
Kgr2	1.361	1.367	1.299 – 1.375
Kkr2	2.325	2.070	1.972 – 2.872
$E50_2r2$ (mg/L)	37.795	39.150	28.819 – 43.860
α_{r2}	19.815	25.227	7.259 – 29.675
H1s	3.794	4.801	2.726 – 4.996

H2s	3.347	3.923	0.735 – 4.967
H1r1	2.160	2.488	1.205 – 2.831
H2r2	2.776	2.913	0.883 – 3.942

632 Table 1: Parameter values estimates with 95% credibility interval from HFIM PKPD model. V =
633 Volume of distribution; C = clearance, Kg = bacterial growth constant; Kk = bacterial kill
634 constant; E50 = Concentration of drug achieving 50% of efficacy; α = interaction parameter; H =
635 Hill constant. Parameter suffices are defined as follows; 1 = relating to fosfomycin; 2 = relating
636 to amikacin; s = relating to wildtype bacterial population; r1 = relating to 'fosfomycin resistant'
637 bacterial population; r2 = relating to 'amikacin resistant' bacterial population.
638

Strain Number	Species	Resistance mechanisms	Amikacin MIC	Fosfomycin MIC
ST195	*E. coli*	CTX-M-14	4	1
I1057	*E. coli*	CTX-M-15, CMY-23, FQ-resistant	32	2
NCTC 13451	*E. coli*	CTX-M-15, OXA-1, TEM-1, aac6'-Ib-cr, mph(A), catB4, tet(A), dfrA7, aadA5, sulI	16	4
BAA2523	*E. coli*	OXA-48	4	8
L75546	*K. pneumoniae*	NS	64	4
1237221	*K. pneumoniae*	SHV-OSBL, CTX-M-15	8	32
1216477	*K. pneumoniae*	SHV-OSBL, TEM-OSBL, CTX-M-15	8	32
NCTC 13438	*K. pneumoniae*	KPC3	32	32
1256506	*K. pneumoniae*	SHV-OSBL; TEM-OSBL; CTX-M-2; CMY-2	2	128
L41464	*K. pneumoniae*	NS	16	128

639

640 Table 2: Details of strains used in HFIM testing physiological pharmacokinetics of

641 fosfomycin/amikacin. NS = not sequenced, at time of writing.

Fosfomycin MIC (mg/L)	Amikacin MIC (mg/L)								
	1	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
256	91.33%	51.81%	3.43%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
128	99.42%	91.33%	51.81%	3.43%	0.00%	0.00%	0.00%	0.00%	0.00%
64	99.97%	99.42%	91.33%	51.81%	3.43%	0.00%	0.00%	0.00%	0.00%
32	100.00%	99.97%	99.42%	91.33%	51.81%	3.43%	0.00%	0.00%	0.00%
16	100.00%	100.00%	99.97%	99.42%	91.33%	51.81%	3.43%	0.00%	0.00%
8	100.00%	100.00%	100.00%	99.97%	99.42%	91.33%	51.81%	3.43%	0.00%
4	100.00%	100.00%	100.00%	100.00%	99.97%	99.42%	91.33%	51.81%	3.43%
2	100.00%	100.00%	100.00%	100.00%	100.00%	99.97%	99.42%	91.33%	51.81%
1	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	99.97%	99.42%	91.33%

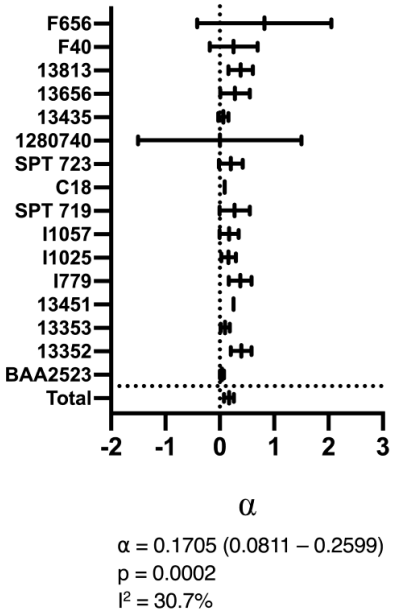
642 Table 3: Probability of attainment of the target $(fAUC_F/\ MIC_F) * (fAUC_A/\ MIC_A) > 2709.5$

643 across a range of amikacin and fosfomycin MICs using 10,000 Monte Carlo simulated neonatal

644 amikacin and fosfomycin *f*AUCs. Grey shading denotes MIC combinations with probability of

645 target attainment < 95%.

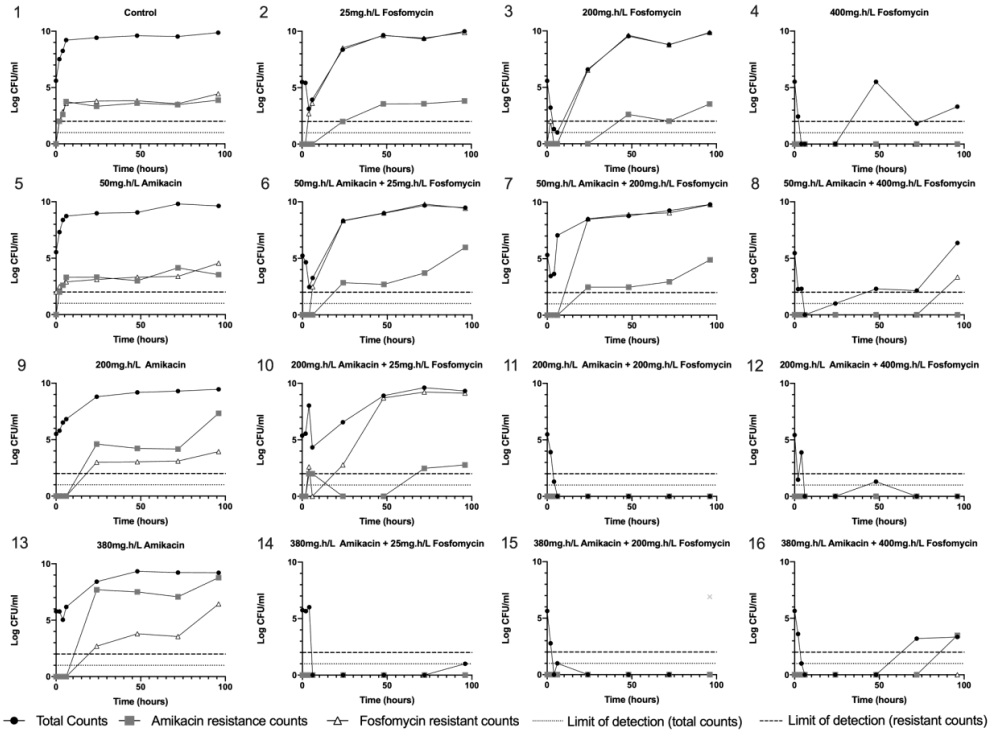
646 **Figures**



647

648 Figure 1– Modelled output for checkerboard assays to three antimicrobial combinations against
649 16 isolates, with a combined total statistic for each combination. α is the interaction parameter
650 in the Greco model indicating the level of synergy. A confidence interval (CI) >0 indicates
651 presence of synergy; CI <0 indicates antagonism; a CI containing 0 indicates no interaction with
652 additive effects only. α and p values for combined statistic are given below the figures. I^2
653 represents the heterogeneity in effect between individual strains.

654



655

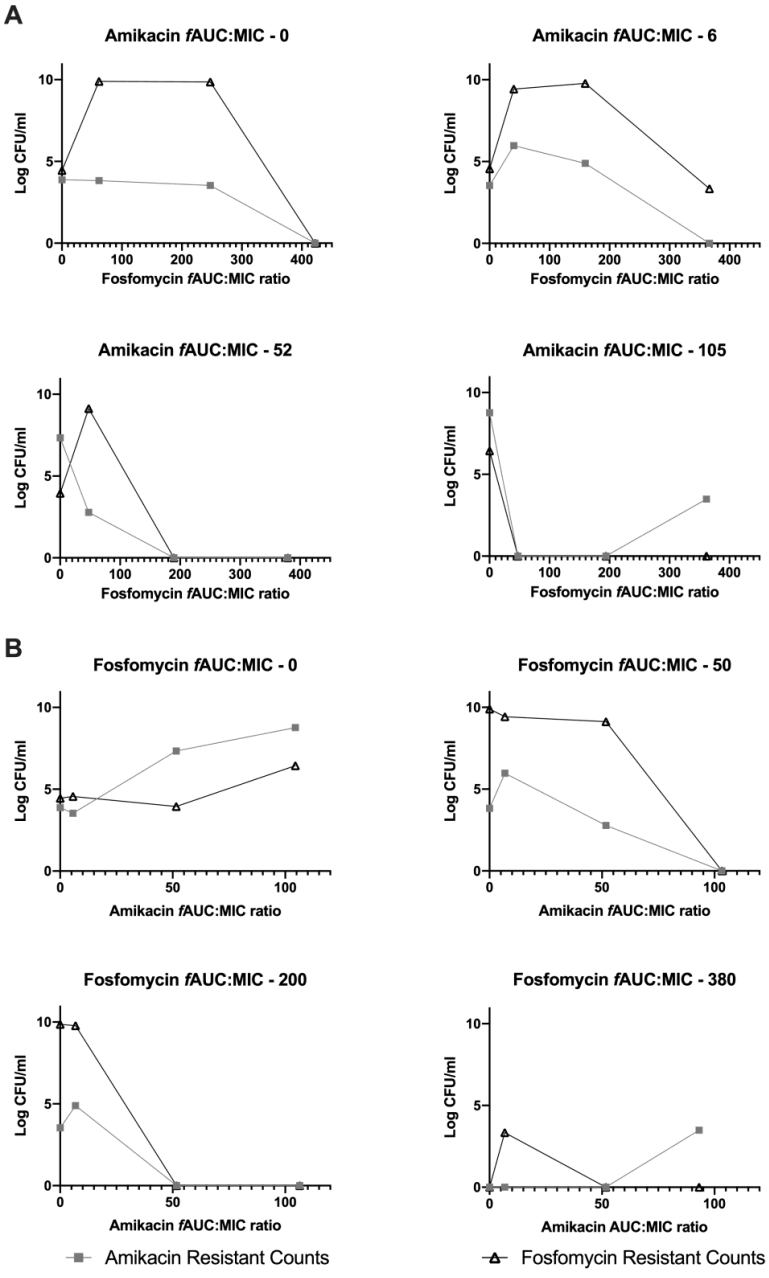
656

657 Figure 2 – Pharmacodynamic output of 16-arm fosfomycin/amikacin combination HFIM

658 experiment, with labelled *f*AUC$_{0\text{-}24}$ for each arm. Grey cross in arm 15 was a real data-point in

659 the initial experiment but was not reproducible in repeat experiments. It is demonstrated here

660 for completeness but was not included in the modelling.



661

662 Figure 3 – Pharmacodynamic relationships of emergence of resistance in relation to modelled

663 *f*AUC:MIC ratios for each agent. (A) Increasing fosfomycin *f*AUC:MIC on a background of fixed

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664 Amikacin *f*AUC:MIC; (B) Increasing amikacin *f*AUC:MIC on a background of fixed fosfomycin

665 *f*AUC:MIC.

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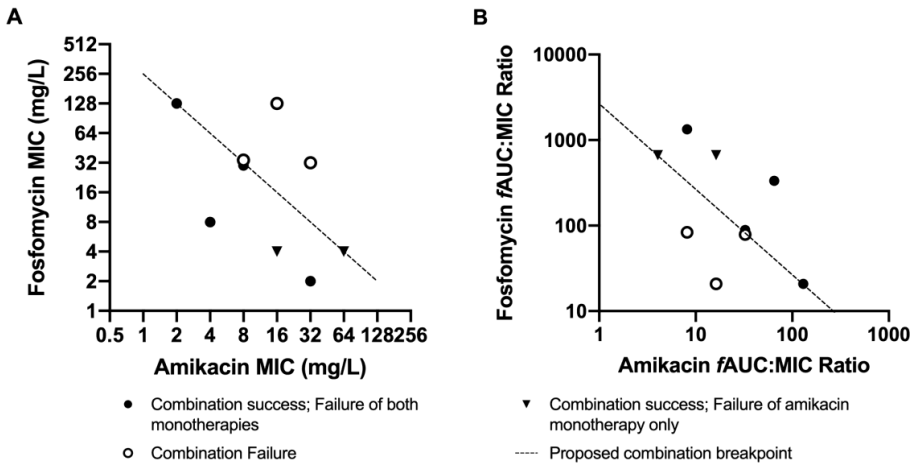
666



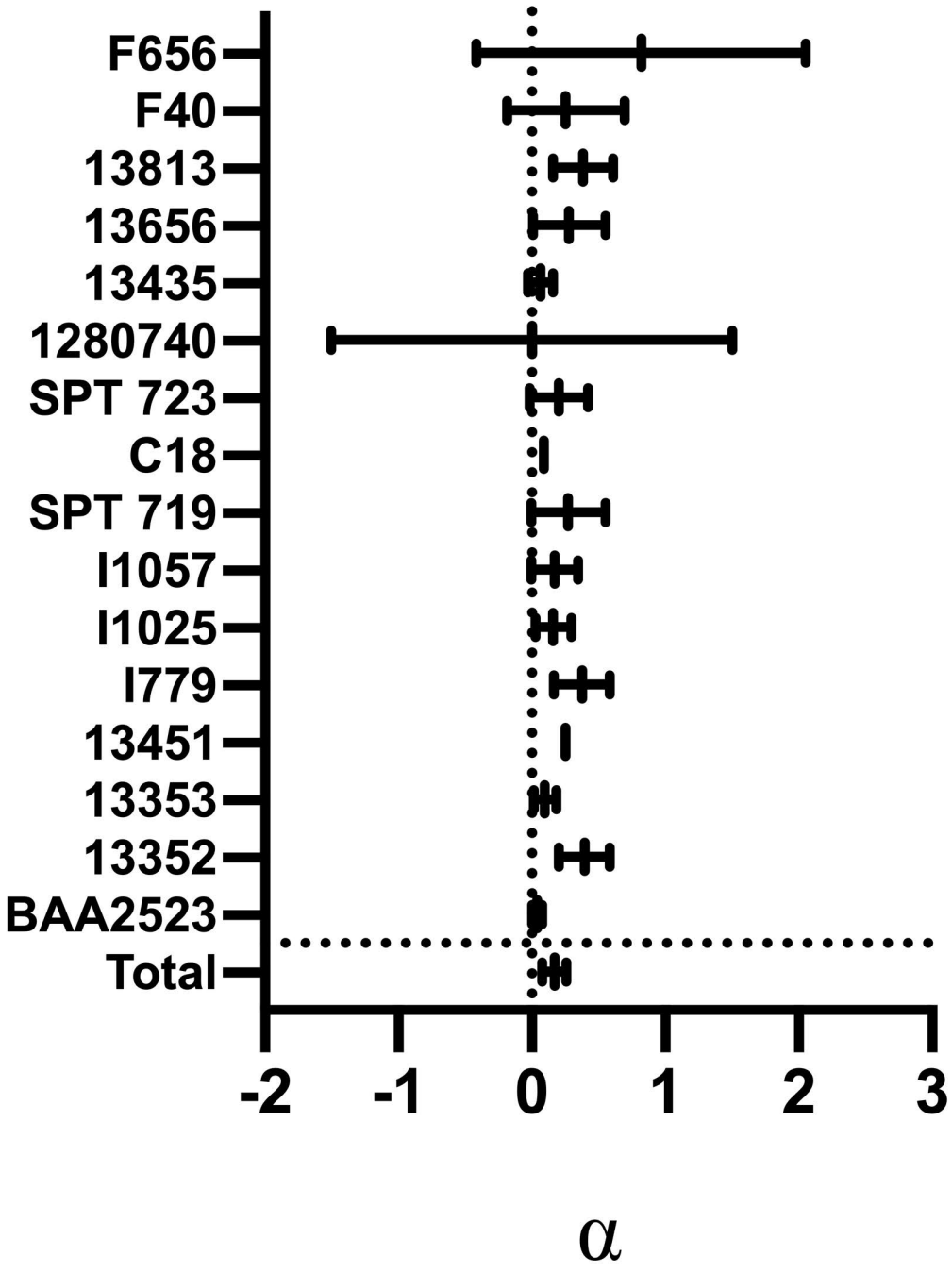
667

668 Figure 4 – Summary of pharmacodynamic outputs of fosfomycin/amikacin antimicrobial

669 combination and monotherapy regimens in HFIM shown by pathogen fosfomycin/amikacin

670 MICs (A) and fosfomycin/amikacin *f*AUC:MIC ratio (B). Success is defined by bacterial kill to

671 sterility at the end of the experiment.



$\alpha = 0.1705\ (0.0811 - 0.2599)$

$p = 0.0002$

$I^2 = 30.7\%$

Antimicrobial Agents and Chemotherapy