Synergistic activity and molecular modelling of fosfomycin combinations with some antibiotics against multidrug resistant \textit{Helicobacter pylori}

Ahmed Megahed Abouwarda$^1$ · Tarek Abdelmonem Ismail$^1$ · Wael Mohamed Abu El-Wafa$^1$ · Ahmed Hassan Ibrahim Faraag$^2$

Received: 12 February 2022 / Accepted: 14 April 2022 / Published online: 29 April 2022
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Abstract
Antibiotic resistance represents the main challenge of \textit{Helicobacter pylori} infection worldwide. This study investigates the potential bactericidal effects of fosfomycin combinations with clarithromycin, metronidazole, ciprofloxacin, amoxicillin, rifampicin, and doxycycline against thirty-six \textit{H. pylori} strains using the checkerboard and time-kill assay methods. The results showed that $\geq 50\%$ of the strains were resistant to the six antibiotics. Remarkably, only six strains exerted resistance to these antibiotics, with the minimum inhibitory concentrations (MICs) ranges of (3.2–12.8 mg/l), (32–256 mg/l), (3.2–51.2 mg/l), (3.2–25.6 mg/l), (1.6–3.2 mg/l), and (25.6 > 51.2 mg/l), respectively. The seven antibiotics were evaluated through in silico studies for their permeability and ability to bind UDP-N-acetylglucosamine1-carboxyvinyltransferase (MurA) of \textit{H. pylori}. The results indicated that fosfomycin exhibited the highest predicted membrane permeability (membrane $\Delta G_{\text{insert}} = -37.54$ kcal/mol) and binding affinity (docking score = $-5.310$ kcal/mol) for \textit{H. pylori} MurA, compared to other tested antibiotics. The combinations of fosfomycin with these antibiotics exerted synergistic interactions (Fractional inhibitory concentration, FIC index < 1) against the six strains. Importantly, the combinations of fosfomycin with clarithromycin, doxycycline and rifampicin achieved bactericidal effects (reduction $\geq 3.0 \log_{10} \text{cfu/ml}$) against the most resistant \textit{H. pylori} strain. Notably, these effects increased with presence of metronidazole, which enhanced the activity of the fosfomycin combination with amoxicillin from a weak inhibition to bactericidal effect. This study provides evidence that the combination of fosfomycin with either clarithromycin, amoxicillin, doxycycline, or rifampicin (especially with the presence of metronidazole) could be a promising option for treating MDR \textit{H. pylori} infection.

Keywords \textit{Helicobacter pylori} · Fosfomycin · Metronidazole · Clarithromycin · Resistant · Synergism · Docking

Introduction
\textit{Helicobacter pylori} is a Gram negative, microaerophilic, motile, and spiral-shaped bacterium. It represents one of the most frequent bacterial human infections worldwide (Lien et al. 2019). This clinically-important bacterium is linked to many gastrointestinal diseases, including gastritis, peptic ulcer, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma. Additionally, there were extragastric diseases associated with \textit{H. pylori} infection, including cardiovascular, respiratory, extra-gastrudodenal digestive, neurological, dermatological, autoimmune and growth disorders (Flores-Treviño et al. 2018; Gravina et al. 2018).

There are more than 4.4 billion patients worldwide estimated to have \textit{H. pylori} infection (Hooi et al. 2017), which significantly influenced by age, sex, geographical regions,
ethnicity, and socio-economic factors (Reffert and Smith 2014; Hooi et al. 2017; Zamani et al. 2018; Hu et al. 2020). Meanwhile, Africa has the highest rate of H. pylori infection worldwide, followed by South America and Western Asia, with prevalence of 70.1, 69.4% and 66.6%, respectively (Reffert and Smith 2014). In Egypt, the prevalence of H. pylori infection ranged from 60 to 90% (Mohamed et al. 2014; El-Khlousy et al. 2016; Ismail and Mostafa 2018).

Currently, antibiotic resistance is the main challenge in the management of H. pylori infection worldwide. The recent systematic reviews and meta-analyses demonstrated that the primary and secondary resistance rates to clarithromycin (CLA), metronidazole (MET) and ciprofloxacin (CIP) exceeded 15% (alarming levels) in all developed and developing countries (Safavi et al. 2016; Gong and Yuan 2018; Savoldi et al. 2018; Lien et al. 2019; Hu et al. 2020). It is noteworthy to mention that Africa had the second rates of amoxicillin (AM) and doxycycline (DO) resistance in H. pylori infection worldwide, with a prevalence rate of 65.5 and 43.9%, respectively (Arslan et al. 2017). Despite, the global rifampicin (RIF) resistance in H. pylori is limited, the infection with rifampicin-resistant H. pylori has significantly increased in some geographical regions; America, Europe and Oceania, with a prevalence rate of 46.1, 33.3 and 23.1%, respectively (Flores-Treviño et al. 2018). Thus, alternative safe and effective treatment regimens for resistant H. pylori infection are urgently needed.

Fosfomycin (FOS) is a broad-spectrum antibiotic, with putative activity against multidrug resistant (MDR) Gram-positive and Gram-negative pathogens. It inhibits the early stage of the bacterial cell wall synthesis. Several studies have investigated the synergistic effects of FOS when combined with other antibiotics that act via a different mechanism of action, thereby allowing for decreased dosages and lower toxicity (Zhan et al., 2018; Davis et al., 2020; Abu El-Wafa and Ibrahim, 2020; Seok et al., 2020). In this study we investigated the potential bactericidal effects of FOS combinations with antibiotics (CLA, CIP, AM, DO and RIF) against MRD H. pylori in the presence and absence of MET.

**Materials and methods**

**Bacterial strains and growth conditions**

The H. pylori strains (n = 36) used in this study were previously isolated from gastric biopsy specimens from patients with gastric and peptic ulcer. The strains were previously identified based on standard biochemical and molecular (16S rRNA) approaches (Mostafa et al. 2018). The H. pylori cultures were separately preserved with 50% (v/v) glycerol at −70 °C until use. All the experiments of this study were carried out under microaerophilic conditions using an anaerobe jar (Oxoid, Ltd) with microaerophilic gas-generating kit (code no. BR 56; Oxoid, Ltd).

**Antibiotic susceptibility testing**

The susceptibility of thirty six H. pylori strains to FOS, CLA, MET, CIP, AM, RIF and DO (European pharmacopoeia reference standards) was evaluated through the determination of MIC using agar plate dilution method according to European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2020). Briefly, twofold serial dilutions of these antibiotics were separately performed in Mueller–Hinton agar (MHA, Oxoid) plates supplemented with 5% defibrinated sheep blood. Each agar plate was inoculated with 2 μl /spot of each H. pylori inoculum (1 × 10⁶ cfu/ml). The final concentrations of FOS and MET were ranging from 0.5 to 512 mg/l, whereas the final concentrations of CLA, CIP, AM, RIF and DO were ranging from 0.00315 to 51.2 mg/l. Following the inoculation, the plates were dried at room temperature and then incubated at 37 °C for 4 days. The MIC was defined as the lowest concentration that inhibited visible growth of bacteria.

**Homology modelling**

The homology model of UDP-N-acetylglucosamine 1-carboxyvinyltransferase (MurA) sequence was performed using the SWISS-MODEL prediction tools (https://swissmodel.expasy.org/). A template search with BLAST and HHblits has been performed against the SWISS-MODEL template library based on the ProMod3 target template alignment and evaluated using the QMEAN score function (Guex et al. 2009; Benkert et al. 2011; Remmert et al. 2012; Bienert et al. 2017; Bertoni et al. 2017; Waterhouse et al. 2018) The quaternary structure and InterPro protein families and domains tool analysis of MurA of the target H. pylori sequence (UniProtKB-ID: P56189) were used to build the three dimensional (3D) model with template sequence of high sequence identity according to (Kessler et al. 1982; Bertoni et al. 2017; Blum et al. 2021).

**In Silico Docking Study**

The docking experiment was performed with Glide’s Extra Precision (XP) program from Schrödinger 16.4 (Friesner et al. 2006). The analysis was conducted using the following ligands: FOS, CLA, MET, CIP, AM, RIF and DO which were retrieved from PubChem Bioassay. Maestro 11.9 and LigPrep 2.4 applications have been used for the preparation of the ligand. For the docking analysis, MurA homology model of the crystallographic structure was used. Figure 1 revealed the 3D structure of MurA. The grid size was defined as 20 Å by default for each protein. The MacroModel of
Schrödinger software was used to reduce energy for all ligands (Jorgensen et al. 1996; Kaminski et al. 2001; Schrödinger 2013). The pKa value of each ionizable atom in FOS was determined by utilizing the empirical pKa panel of Schrödinger software. Additionally, the generation of the most likely ionized and tautomerized states of FOS in different pH levels ranging from 3 to 11 was also evaluated (Balogh et al. 2012; Schrödinger 2013).

**In silico models for predicting membrane permeability**

Computationally, membrane permeability prediction of FOS, CLA, MET, CIP, AM, RIF and DO using the membrane permeability prediction tools in the physics-based permeability prediction module within the Schrödinger’s Small-Molecule Drug Discovery Suite 12.8 based on Membrane ∆G Insert Eq. (1) (Rezai et al. 2006; Leung et al. 2016; Schrödinger Release 2019–1 2019). Energy minimization for all ligands was performed using the macro-Model of Schrödinger’s software (Jorgensen et al. 1996; Kaminski et al. 2001; Schrödinger 2013).

\[
\text{Membrane } \Delta G \text{ Insert} = \text{energy of Membrane HDLD} + \text{Membrane State Penalty}
\]

**Determination of fractional inhibitory concentrations**

The FIC of double combinations of FOS with CLA, CIP, AM, RIF, DO and MET against *H. pylori* strains was determined by checkerboard microdilution method (Kim et al. 2016). Briefly, FOS was serially diluted twofold in a horizontal orientation, whereas CLA, CIP, AM, RIF, DO and MET were serially diluted twofold in a vertical orientation. The final concentrations of FOS or MET in 200 µl Mueller–Hinton broth (MHB) were ranging from 512 to 1.0 mg/l, whereas the final concentrations of CLA, CIP, AM, RIF and DO in 200 µl MHB were ranging from 5.12 to 0.00315 mg/l. The inoculum size of each test strain was approximately 1 × 10^6 cfu/ml. Inoculated and un-inoculated wells (containing 200 µl MHB) were considered as positive and negative controls, respectively. Following the inoculation, the plates were incubated for 48 h at 37 °C. Additionally, FICs of triple combinations of FOS/MET with CLA, CIP, AM, RIF and DO were determined by the same above-mentioned method. FIC index of the combinations was calculated by the sum of the FIC of each antibiotic alone (MIC of antibiotic in combination/
MIC of antibiotic alone). FIC index of antibiotic combinations defined as synergy ($\Sigma$FIC ≤ 1), indifference (1.0 < $\Sigma$FIC ≤ 4) or antagonism ($\Sigma$FIC > 4) (Kamatou et al. 2006). The MICs of the synergistic antibiotic combinations were further tested against the representative strain by a time-kill assay.

**In vitro time kill assays**

The bactericidal activities of antibiotics (MET, CLA, CIP, AM, RIF and DO) and their respective combinations with FOS (in the presence/absence of MET) against the representative strain were evaluated by performing time-kill assay (Coudron and Stratton 1995). Briefly, single antibiotics, double and triple antibiotic combinations were performed in sterile MHB, and then inoculated with 10 µl of 48 h culture of test strain. The final inoculum size of each test strain in 50 ml MHB was 1 × 10^6 cfu/ml. Aliquots were taken at different time intervals (0, 3, 6 and 24 h) and serial tenfold dilutions were prepared in sterile sodium chloride solution (0.9%, w/v) as needed. Three replicates of each diluent were spotted on MHA supplemented with 5% defibrinated sheep blood, dried at room temperature and then incubated 4 days at 37 °C (Inoculated and un-inoculated MHB were considered as positive and negative controls). The data were analyzed by using mean colony counts (Log_{10} cfu/ml) from the replicates of each diluent at each time interval. The limit of quantification was 2 Log_{10} cfu/ml. The synergy of the combination was defined as a 2 Log_{10} cfu/ml decrease compared with the most active antibiotic in this combination, whereas the bacteriostatic and bactericidal effects were defined as 2 and 3 Log_{10} cfu/ml decrease relative to the initial inoculum, respectively.

**Results**

The susceptibility of 36 *H. pylori* strains to six different antimicrobial agents was estimated through the determination of the minimum inhibitory concentration (MIC) using the agar plate dilution method. As shown in Fig. 1, 24 (66.67%) strains were resistant to CLA, 23 (63.89%) strains were resistant to CIP, 21 (58.33%) strains were resistant to AM, 19 (52.78%) strains were resistant to DO and MET, and 18 (50%) strains were resistant to RIF. Remarkably, six *H. pylori* strains were found resistant to the six tested antibiotics.

The MICs of the seven different antibiotics against the six MDR *H. pylori* strains are summarized in Table 1. The results showed that all strains were resistant to CLA, MET, CIP, AM, RIF and DO. No interpretive criteria are provided for FOS on *H. pylori* in either the CLSI or the EUCAST. The MICs ranges of the six antibiotics against the test strains were (3.2–12.8 mg/l), (32–256 mg/l), (3.2–51.2 mg/l), (3.2–25.6 mg/l), (1.6–3.2 mg/l), and (25.6–> 51.2 mg/l), respectively. The MIC of FOS against these strains was ranging from 128 to 256 mg/l. Additionally, all the test strains were classified as MDR since they exhibited resistance to more than two antibiotics related to different antibiotic categories. Notably, *H. pylori* HP-1 exhibited the highest MIC values of all tested antibiotics. Thus, this strain was selected as a representative strain for time kill studies.

The 3D structure of *H. pylori* MurA was determined based on the principle of homology modeling, using of a templet (PDB:5UJS) from *Campylobacter jejuni* ATCC 700,819. The structure analysis indicated that *H. pylori* MurA showed 60.05% sequence identity with crystal structure of MurA protein from *Campylobacter jejuni* ATCC 700,819. Additionally, the InterPro protein families and domains of *H. pylori* MurA demonstrated that the MurA possesses one active site contain Cys^{117} and 3 binding sites contain Arg^{91}, Asp^{308} and Leu^{330}, respectively (Figs. 2, 3).

| Strains  | MIC of antibiotics (mg/l)/ Susceptibility pattern* | Resist-ance pattern |
|---------|--------------------------------------------------|--------------------|
| HP-1    | 12.8/R 25.6/R > 51.2/R 51.2/R 3.2/R 256/R 256/NL | MDR                |
| HP-2    | 3.2/R 6.4/R > 51.2/R 3.2/R 1.6/R 256/R 128/NL | MDR                |
| HP-3    | 12.8/R 3.2/R > 51.2/R 3.2/R 3.2/R 64/R 256/NL | MDR                |
| HP-4    | 6.4/R 25.6/R 25.6/R 25.6/R 3.2/R 128/R 128/NL | MDR                |
| HP-5    | 3.2/R 12.8/R 25.6/R 25.6/R 3.2/R 128/R 128/NL | MDR                |
| HP-6    | 3.2/R 3.2/R > 51.2/R 3.2/R 3.2/R 32/R 128/NL | MDR                |

*According to EUCAST, MIC minimum inhibitory concentration, R resistant, NL not listed in EUCAST guideline, MDR multidrug resistant, FOS fosfomycin, MET metronidazole, CLA clarithromycin, CIP ciprofloxacin, AM amoxicillin, DO doxycycline, RIF rifampicin
The molecular docking study of the seven tested antibiotics to *H. pylori* MurA indicated that FOS exhibited the highest binding affinity with protein active site of *H. pylori* MurA, followed by DO, with docking scores equal to −5.310 and − 5.135 kcal/mol, respectively. Whereas, CIP, MET, and AM showed moderate binding affinity, with docking scores equal to −4.744, − 4.549, and − 4.356 kcal/mol, respectively. The lower binding affinity with protein active site of *H. pylori* MurA was observed with CLA and RIF, with docking scores equal to − 3.887, and − 3.834 kcal/mol, respectively (Table 2 and Fig. 4a–g).

The hydrogen (H) bonding interactions in the best docking are also described in Table 2 and Fig. 4a–g. The results showed that the maximum total number of the hydrogen (H) bonds between tested antibiotics and the protein active site of *H. pylori* MurA was observed with CIP, which forms 5 H-bonds; 3H bonds with Arg^{236} and 2H bonds with Lys^{300} and Glu^{327}, with bond lengths of 1.98, 2.02, 2.52, 2.12 and 1.80 Å, respectively, followed by FOS and RIF, which form 4H bonds; FOS forms 2H bonds with Glu^{190}, and 2H bonds with Arg^{236} and Thr^{307}, with bond lengths of 1.54, 1.71, 2.11 and 2.61 Å, respectively, REF forms 2H bonds with Thr^{352} and 2H bonds with Lys^{300} and Glu^{327}, with bond lengths of...
The Docking scores and hydrogen bonds between legends and H. pylori MurA

| Ligands | Free energy of binding (Kcal/mol) | Residues involved in hydrogen bonding | H-bonds distance (Å) | Number of hydrogen bonds |
|---------|----------------------------------|--------------------------------------|----------------------|--------------------------|
| FOS     | −5.31                            | Glu\(^{190}\) (B), Arg\(^{236}\) (B), Thr\(^{307}\) (B) | 1.54, 1.71, 2.11, 2.61 | 4H bonds                |
| DO      | −5.14                            | Glu\(^{190}\) (B), Asp\(^{308}\) (B) | 1.62, 2.13, 1.60       | 3H bonds                |
| CIP     | −4.74                            | Arg\(^{236}\) (B), Lys\(^{300}\) (B), Glu\(^{332}\) (B) | 1.98, 2.02, 2.52, 2.12, 1.80 | 5H bonds                |
| MET     | −4.55                            | Glu\(^{190}\) (B), Thr\(^{307}\) (B) | 2.26, 1.85             | 2H bonds                |
| AM      | −4.36                            | Glu\(^{190}\) (B), Arg\(^{334}\) (B) | 1.72, 1.94             | 2H bonds                |
| CLA     | −3.89                            | Lys\(^{300}\) (B), Thr\(^{300}\) (B), Arg\(^{334}\) (B) | 2.23, 1.87, 2.16       | 3H bonds                |
| RIF     | −3.83                            | Lys\(^{300}\) (B), Glu\(^{327}\) (B), Thr\(^{352}\) (A) | 2.33, 1.71, 2.39, 2.66 | 4H bonds                |

Amino acids form 2 & 3 hydrogen bonds are highlighted in italic & bold, respectively

FOS fosfomycin, MET metronidazole, CLA clarithromycin, CIP ciprofloxacin, AM amoxicillin, DO doxycycline, RIF rifampicin; Arg arginine, Asp aspartic, Glu glutamic acid, Lys lysine, Thr threonine

(A, B) protein chains A & B, respectively

2.33, 1.71, 2.39 and 2.66 Å, respectively. Whereas, DO and CLA form 3H bonds; DO forms 2H bonds with Glu\(^{190}\) and H bond Asp\(^{308}\), with bond lengths of 1.62, 2.13 and 1.60 Å, respectively, CLA forms 3H bonds with Lys\(^{300}\), Thr\(^{300}\) and Arg\(^{334}\), with bond lengths of 2.23, 1.87 and 2.16 Å, respectively. The lower number of H-bonds between tested antibiotics and the protein active site of H. pylori Mur A was observed with MET and AM, which forms 2 H-bonds; MET forms 2H bond with Glu\(^{190}\) and Thr\(^{307}\), with bond lengths of 2.26 and 1.85 Å, respectively, AM forms 2H bonds with Glu\(^{190}\) and Arg\(^{334}\), with bond lengths of 1.72 and 1.94 Å, respectively.

The effect of different pH levels on the docking score of FOS are summarized in Table 3 and Figs. 5, 6a-c. The obtained results revealed that the docking score of FOS was increased under alkaline conditions, reaching −7.456 kcal/mol in the pH values ranging from 6 to 3, and under acidic conditions, the docking score of FOS deceased to −6.04 and −5.67 kcal/mol (Fig. 7 and Table 4).

The interactions of FOS combinations with different antibiotics against the six test strains are summarized in Table 5. The results indicated that all the examined combinations exhibited good synergistic activities FIC index < 1 and re-sensitized the test strains to the used antibiotics. Notably, 128 mg/l was the optimal concentration of FOS for synergetic interactions (FIC index < 1) with other antibiotics against HP-1 and HP-3, whereas 64 mg/l of FOS was the optimal concentration for the same interactions against the other four test strains. Interestingly, MICs of CLA, AM, CIP and DO in double FOS combinations were decreased from the ranges (3.2–12.8 mg/l), (3.2–25.6 mg/l), (3.2–51.2 mg/l) and (3.2> 51.2 mg/l) to the ranges (0.0125–0.025 mg/l), (0.0125–0.05 mg/l), (0.025–0.05 mg/l) and (0.025–0.05 mg/l), respectively.

Data on the time-kill kinetics of the tested single and combined antibiotics were consistent with those of the checkerboard experiments. The time-kill kinetics of antibiotics (CLA, MET, CIP, AM, RIF and DO) and their respective combinations with FOS against most resistant strain (HP-1) are presented in Fig. 8. As shown, kinetics of all single antibiotics against the representative strain were similar to those of the control, except for FOS, which caused an initial reduction in bacterial count within 3 h of post-treatment, followed by a considerable regrowth similar to the control after 6 h of treatment and lasted up to 24 h. Additionally,
Fig. 4  

a. The interaction between *Helicobacter pylori* Mur A and fosfomycin.  
b. The interaction between *Helicobacter pylori* Mur A and doxycycline.  
c. The interaction between *Helicobacter pylori* Mur A and ciprofloxacin.  
d. The interaction between *Helicobacter pylori* Mur A and metronidazole.  
e. The interaction between *Helicobacter pylori* Mur A and amoxicillin.  
f. The interaction between *Helicobacter pylori* Mur A and clarithromycin.  
g. The interaction between *Helicobacter pylori* Mur A and rifampicin.  
Arg arginine, Glu glutamic acid, Lys lysine, Thr threonine, Phe phenylalanine, Pro proline, Arg arginine, Asp aspartate, Val valine, Ser serine, Tyr tyrosine, Leu leucine, Asn asparagine, Gly glycine, Ile isoleucine, the blue dashed lines represent H-bonds and the numbers denote the distance of the H-bonds.
FOS combinations with AM, CIP and MET exhibited an initial reduction within 3–6 h post-treatment, followed by a considerable regrowth similar to the control after 24 h of treatment.

Figure 8 also shows that the combinations of FOS with CLA, DO and RIF produced bacteriostatic effects after 6 h of treatment, with 2.2, 2.1 and 2.08 log_{10} reduction in bacterial count, respectively. Moreover, these combinations
exhibited bactericidal effects after 24 h post-treatment, with 3.2, 3.8 and 3.18 Log_{10} cfu/ml reduction in the initial inoculum, respectively. Notably, MET improved the activity of the combination of FOS with AM against HP-1 from a weak inhibition to a bacteriostatic effect within 3 h of treatment, with a reduction of 2.28 log_{10} cfu/ml, followed by bactericidal effects after 6 h of treatment and lasted up to 24 h, with a reduction of 3.28 and 3.56 log_{10} cfu/ml, respectively. Additionally, MET enhanced the bactericidal activity of FOS combinations with CLA, RIF and DO against the representative strain 24 h post-treatment, with the reduction in bacterial count increasing from 3.2, 3.18 and 3.6 to 4.04, 4.09 and 4.02 log_{10} cfu/ml, respectively. On the other hand, the presence of MET did not influence the activity of FOS/CIP combination against HP-1 (Fig. 2A–E). To the best of our knowledge, no previous study have investigated the bactericidal effects of these combinations against MDR H. pylori strains.

### Discussion

The effectiveness of standard therapeutic regimens for *H. pylori* infection has drastically reduced in recent years due to the increasing emergence of antibiotic resistance and the side effects of these regimens. Thus, new therapeutic options are urgently needed to combat the emergence of MDR *H. pylori* infections. Enhancing the efficacy of old antimicrobial agents represents one of the most feasible solutions for overcoming the high prevalence of MDR strains. In this study, we evaluate the synergistic potential of FOS combinations with a series of antibiotics used as first and second lines for the treatment of *H. pylori* infections. Additionally, the activity of these combinations against MDR *H. pylori* strains was also evaluated in the presence of MET.

Fig. 5 Effect of different pH levels on the total charges of FOS
failure of *H. pylori* eradication therapy is mainly due to the massive use of wasn’t for treating parasite infestations and uncontrolled consumption of macrolide and fluoroquinolones antibiotics in developing countries (Mégraud 2004; Kuo et al. 2017; Savoldi et al. 2018). Additionally, Klein and his co-worker reported that between 2000 and 2015, antibiotic consumption, expressed in defined daily doses, has increased 65% (Klein et al. 2018).

Fosfomycin is a bactericidal analog of phosphoenolpyruvate that has been previously been employed for uncomplicated urinary tract infections. The role of this antibiotic has been recently gained interest among physicians worldwide and the world health organization (WHO) defined it as critically important due to its potential efficacy against MDR Gram-positive and Gram-negative bacteria (Zdziebło et al. 2014; Falagas et al. 2016; Ruiz Ramos and Salavert Lletí 2019; Williams 2020). Additionally, many investigations mentioned that FOS may prove to be useful for *H. pylori* infection when the first-line antibiotic regimens fail (Barahona-Garrido et al. 2013; Boyanova et al. 2014; Falagas et al. 2016).

The docking results of the present study demonstrated that FOS had the highest binding affinity (docking score = −5.310 kcal/mol) for *H. pylori* MurA, in comparison to the other tested antibiotics (CLA, MET, CIP, AM, RIF and DO), which used as the first and second lines for the treatment of *H. pylori* infections. Furthermore, FOS binds the protein active site of *H. pylori* MurA by forming 4 H bonds with Glu190, and 2H bond with Arg234, and Thr329 and two salt bridges with Arg334 as it possess two negative charge, on the other hand at pH value of 7, the binding affinity was −5.708 kcal/mol with the formation of 3 H-bonds with Arg334, and Thr329 and one salt bridges with Arg234 as it possess one negative charge. However at pH value range from 6 to 3, the docking score was 2.945 kcal/mol which mean very low binding affinity with the formation of 3 H-bonds with Arg334, and Asp308 under non ionized state.

Generally, most of antibiotics need to pass through at least one cellular membrane of Gram-negative bacteria to reach their intended target. Although tight binding of an antibiotic to its intended target is important for potency, poor membrane permeability often led to decrease the concentration of antibiotic inside the bacterial cell and reduce its efficacy (Wolak and Thorne 2013; Bennion et al. 2017; Domalaon et al. 2018). Interestingly, our *In silico* data showed that FOS had the highest membrane permeability (membrane ∆G insert = −37.54 kcal/mol) compared to other tested antibiotics, which exhibited low membrane permeabilities, with ∆G Insert ranging from −5.67 to −31.26 kcal/mol. From these findings, which agree with previous studies (Barahona-Garrido et al. 2013; Boyanova et al. 2014; Falagus et al. 2016), FOS could be a good suggestion as antimicrobial agent against MDR *H. pylori*, especially when the first-line antibiotic regimens fail.

In this study, the combinations of FOS with other tested antibiotics (CLA, MET, AM, CIP, RIF and DO) showed good synergistic effects (FIC index < 1) against all *H. pylori* strains and decreased the MICs of these antibiotics lower than the susceptible breakpoint. These findings obviously indicated that FOS might be adequate to re-sensitize the MDR *H. pylori* to these antibiotics in suitable combinations. The interaction between FOS and these antibiotics against MDR *H. pylori* was only investigated by one previous study, which supported our findings regarding the synergistic effects of FOS combinations with CLA, MET and AM against *H. pylori* strains (Blacky et al. 2005). Generally, our results are consistent with those reported by previous studies, which revealed that FOS/CIP combinations achieved synergistic effects against MDR strains of other Gram negative bacteria such as *Klebsiella pneumonia* (Yu et al. 2017), *Pseudomonas aeruginosa* (Walsh et al. 2016) and *E. coli* (Abu El-Wafa and Ibrahim 2020).

The data of time-kill curves of the single and combinations of used antibiotics were consistent with those of the checkerboard experiments. Time-kill curves of single antibiotics (FOS, CAL, MET, CIP, AM, RIF and DO) against representative strain (HP-1) showed a considerable regrowth
similar to control after 24 h of post-treatment. Additionally, FOS combinations with AM, CIP and MET exhibited an initial reduction within 3–6 h post-treatment followed by a considerable regrowth similar to control after 24 h of post-treatment. These findings were in agreement with those mentioned by previous studies, which revealed that the regrowth phenomenon might be due to that the total bacterial burden contained two particular subpopulations with

Table 4  Computational exploration of the membrane permeability of H. pylori for seven different antibiotics

| Ligands | Membrane permeability prediction |
|---------|---------------------------------|
| Membrane Insert (kcal/mol) | Membrane HDLD (kcal/mol) | Membrane GB (kcal/mol) | Membrane State Penalty (kcal/mol) | Log Perm RRCK (cm/s) |
| FOS | −37.54 | −29.87 | −6.15 | −7.67 | −6.34 |
| AM | −31.26 | −24.45 | −10.30 | −6.81 | −6.39 |
| DO | −29.85 | −21.86 | −9.54 | −7.99 | −6.58 |
| RIF | −17.08 | −15.11 | −7.06 | −1.97 | −6.21 |
| CIP | −13.84 | −10.33 | −4.40 | −3.50 | −5.33 |
| MET | −6.04 | −6.04 | −5.02 | 0.00 | −4.38 |
| CLA | −5.67 | −3.61 | −6.09 | −2.07 | −5.68 |

1 Membrane ∆G Insert: the total free energy penalty for the ligand to change state and enter the membrane. This is the net of the energy of Membrane HDLD and Membrane State Penalty; 2 Membrane HDLD: the free energy penalty for the neutral form of the ligand in its conformation inside the membrane to enter the membrane (i.e., move from the high dielectric region to the low dielectric region, hence HDLD). 3 Membrane GB: an implicit membrane generalized Born theory model closely reproduces the Poisson–Boltzmann (PB) electrostatic solvation energy profile across the membrane. 4 Membrane State Penalty: a tautomerization penalty is derived from possible tautomer states and their estimated relative populations. These two processes are combined as a state penalty, ∆G state, that represents the free energy cost for the permeant to adopt a particular neutral, tautomeric form for membrane permeation. RRCK Ralph Russ canine kidney cells: 5 Log Perm RRCK: logarithm of the RRCK permeability in cm/s. This property is optimized to reproduce RRCK permeability assay results, with fitted energy.

FOS fosfomycin, MET metronidazole, CLA clarithromycin, CIP ciprofloxacin, AM amoxicillin, DO doxycycline, RIF rifampicin

*Partition energy “∆G” Insert prediction
Table 5  The fractional inhibitory concentrations of FOS combinations with six different antibiotics against six MDR *H. pylori* strains

| Strains | Double | Triple |
|---------|--------|--------|
|         | FOS/CLA FOS/AM FOS/DO FOS/CIP FOS/RIF FOS/MET | FOS/CLA/ MET FOS/MET FOS/AM/MET FOS/DO/MET FOS/CIP/MET FOS/RIF/MET |
| HP-1    | 128/0.025 (0.50) 128/0.05 (0.50) 128/0.05 (0.50) 128/0.05 (0.52) 128/8 (0.53) 64/0.0125/4 (0.27) | 64/0.0125/4 (0.27) 64/0.025/4 (0.27) 64/0.025/4 (0.27) 64/0.025/4 (0.27) 64/0.025/4 (0.27) |
| HP-2    | 64/0.025 (0.50) 64/0.05 (0.50) 64/0.05 (0.50) 64/0.05 (0.53) 64/8 (0.53) 32/0.0125/4 (0.27) | 32/0.0125/4 (0.27) 32/0.0125/4 (0.27) 32/0.0125/4 (0.27) 32/0.0125/4 (0.27) 32/0.0125/4 (0.27) |
| HP-3    | 128/0.025 (0.50) 128/0.05 (0.50) 128/0.05 (0.50) 128/0.05 (0.52) 128/8 (0.53) 64/0.0125/4 (0.31) | 64/0.0125/4 (0.32) 64/0.025/4 (0.32) 64/0.025/4 (0.31) 64/0.025/4 (0.32) 64/0.025/4 (0.32) |
| HP-4    | 64/0.0125 (0.50) 64/0.0125 (0.50) 64/0.025 (0.50) 64/0.025 (0.52) 64/0.05 (0.56) 32/0.0063/4 (0.28) | 32/0.0063/4 (0.28) 32/0.0063/4 (0.28) 32/0.0063/4 (0.28) 32/0.0063/4 (0.28) 32/0.0063/4 (0.28) |
| HP-5    | 64/0.0125 (0.50) 64/0.0125 (0.50) 64/0.025 (0.50) 64/0.025 (0.52) 64/0.05 (0.57) 32/0.0063/4 (0.28) | 32/0.0063/4 (0.28) 32/0.0063/4 (0.28) 32/0.0063/4 (0.28) 32/0.0063/4 (0.28) 32/0.0063/4 (0.28) |
| HP-6    | 64/0.0125 (0.50) 64/0.025 (0.51) 64/0.05 (0.50) 64/0.05 (0.52) 64/0.05 (0.52) 64/8 (0.75) 32/0.0063/4 (0.38) | 32/0.0063/4 (0.38) 32/0.0063/4 (0.38) 32/0.0063/4 (0.38) 32/0.0063/4 (0.38) 32/0.0063/4 (0.38) |

*FIC* fractional inhibitory concentration, *MIC* minimum inhibitory concentration, *MDR* multidrug resistant, *FOS* fosfomycin, *MET* metronidazole, *CLA* clarithromycin, *CIP* ciprofloxacin, *AM* amoxicillin, *DO* doxycycline, *RIF* rifampicin, *FIC* index of combination

\[(A/B) = \frac{FIC_{\text{antibiotic } A} + FIC_{\text{antibiotic } B}}{MIC_{\text{antibiotic in combination}}/MIC_{\text{antibiotic alone}}},\] synergism (*FIC* index ≤ 1); indifference (1.0 < ∑*FIC* ≤ 4) and antagonism (∑*FIC* > 4)
different susceptibility in which the selective amplification of resistant sub-population take over the preferential killing of the susceptible sub-population at a specified time of interaction (Tam et al., 2005; Sim et al., 2014).

Data in the present study showed that the combination of FOS with CLA, DO, and RIF against HP-1 showed bacteriostatic and bactericidal effects after 6 and 24 h of post-treatment, respectively. Notably, MET enhanced the activity of FOS/AM combination against HP-1 from a weak inhibition to bacteriostatic effect within 3 h post-treatment, followed by bactericidal effects within 6 h post-treatment and lasted up to 24 h. Additionally, MET enhanced the bactericidal activities of FOS combinations with CLS, RIF and DO against the representative strain after 24 h of post-treatment, whereas the activity of FOS/CIP combination against HP-1 wasn’t affected in the presence of MET. To the best of our knowledge, no previous study investigated the bactericidal effects of these combinations against MDR H. pylori strains.

To date, only one study reported the synergistic interactions of FOS combinations with some of these antibiotics (CLA, MET and AM) against MDR H. pylori strains (Blacky et al. 2005). In general, the bactericidal activity of
FOS/RIF combination was only reported against some MDR strains of Gram positive bacteria belonging to Enterococcus faecalis, E. faecium and methicillin-resistant Staphylococcus aureus (Simonetti et al. 2018). The combination of FOS and DO was also reported to exhibit synergistic and bactericidal effects against Enterococcus faecium (Davis et al. 2020).

Conclusion

Based on in silico analysis, we found that FOS exhibited the highest predicted membrane permeability and binding affinity for H. pylori MurA, compared to other tested antibiotics, which used as the first and second lines for the treatment of H. pylori infections. Hence, FOS is potentially a promising antibiotic against H. pylori infection. Additionally, this antibiotic enhances the activity of CLA, DO, RIF and AM against MDR H. pylori by decreasing their MICs to the susceptible breakpoints. Moreover, the combinations of FOS with these antibiotics exert bactericidal effects against MDR H. pylori, especially with the presence of metronidazole.

Thus, the combinations of FOS with CLA, DO, RIF and AM could be a promising option for treating MDR H. pylori infection, especially with the presence of metronidazole.

Funding

Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB). This study was not funded.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

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