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ARTICLE

Non-antibiotic pharmaceutical agents as antibiotic adjuvants

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ABSTRACT The emergence of multidrug-resistant bacteria is a global public health issue, which severely hinders clinicians in providing patients with adequate antimicrobial treatment regimens. The strategy of drug repurposing is an emerging strategy in antimicrobial chemotherapy, during which new pharmacological uses are identified for drugs already approved. The aim of our present study was to assess the adjuvant properties of several existing and widely-used pharmacological agents against bacteria in combination with reference antibiotics. *Staphylococcus aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were selected for our experiments. The minimum inhibitory concentrations (MICs) of the tested compounds were determined using the broth microdilution method, while a MIC reduction assay was performed to ascertain the effect of the tested compounds on the MICs of standard antibiotics (ciprofloxacin and gentamicin). Eight tested compounds (namely atorvastatin, celecoxib, clotrimazole, diclofenac-epolamine, ivermectin, lidocaine, mebendazole and terbinafine) showed antibacterial activity on the tested bacterial strains and several agents presented with various degrees of adjuvant (MIC-reducing) properties. Further experiments involving the screening of additional pharmaceutical compounds for their secondary antibacterial and adjuvant properties are warranted.
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Introduction

The discovery of antibiotics and their subsequent introduction into clinical medicine has been one of the main prerequisites for our current – modern day – healthcare to develop (Gaynes 2017). Previously lethal infections have become manageable, the life expectancy of people worldwide has changed drastically and novel medical interventions (e.g., cancer chemotherapy, invasive surgery, organ transplantation, and neonatology) were made possible (Laxminarayan et al. 2013). Bacterial pathogens have also reacted to the use of these agents and developed various resistance mechanisms to avoid their lethal effects (Nikaido 2009). The development of resistant isolates was to be expected by the laws of Darwinian evolution; however, the misuse and overuse of these agents have catalyzed this process to become a severe health problem in the span of only a few decades (Chang et al. 2015). Currently, the emergence of multidrug-resistant (MDR) bacteria is a global public health issue, which severely hinders clinicians in providing patients with adequate antimicrobial treatment regimens (Gajdács and Albericio 2019; Munita and Arias 2016). Several national and global public health authorities have published reports and estimates on the global impact of antibiotic resistance (World Health Organization 2014). The grimmest predictions may be found in the O'Neill report (from the National Health Service of the United Kingdom), projecting 10 million deaths per year by 2050 and 100 billion USD worth of economic burden (O'Neill 2014). Rice et al. have defined the „ESKAPE" bacteria (including E: *Enterococcus faecium*, S: *Staphylococcus aureus*, K: *Klebsiella pneumoniae*, A: *Acinetobacter baumannii*, P: *Pseudomonas aeruginosa*, E: *Enterobacter* spp., or recently *Enterobacteriaceae*) as the most concerning from the standpoint of clinical impact, both mortality-wise and economically (Rice 2010).

The issue of antibiotic resistance in the 21st century is a three-sided problem: *i)* on one hand, the emerge of drug-resistant mutants against newly developed antibiotics is an inevitable evolutionary process (which is common against any kind of noxious agents), *ii)* while the non-prudent use of antibiotics (e.g., for viral infections or other inappropriate indications) only exacerbates this process; *iii)* the costs of clinical research and the development of novel antibiotics – coupled with the relatively modest returns on investment from these drugs – lead to a shift in the interest of pharmaceutical companies to instead develop drugs for chronic (i.e. more „profitable") illnesses; this has resulted in a „discovery void", with very limited amount

of novel agents receiving marketing authorization since the 2000s (Gajdács 2019; Gajdács et al. 2020). In fact, no new broad-spectrum antibiotics were developed since the introduction of the fluoroquinolones in the 1980s (Darrow and Kesselheim 2014). Without new agents, researchers have investigated alternative strategies to combat bacterial pathogens more effectively (Rios et al. 2016). One of the proposed strategies is combination therapy: while the use of two or more existing antibiotics simultaneously in clinical situations is a controversial topic (with very few verified indications), however, the inclusion of non-antibiotic adjuvants seems to be a promising strategy (Ahmed et al. 2014; Tangdén 2014; Szerencsés et al. 2019). These adjuvants include enzyme inhibitors (e.g., clavulanic acid, a β-lactamase inhibitor), efflux pump inhibitors, modulators of bacterial membrane potential, membrane permeabilizers, inhibitors of bacterial cell-cell communication (quorum sensing) and monoclonal antibodies (Wright 2016; Kealey et al. 2017; Drawz and Bonomo 2010). However, it must be noted that most of these molecules did not receive clinical approval due to their toxicity *in vivo* (Tegos et al. 2011). Recently, the adjuvant properties of existing pharmaceutical compounds have received substantial attention. This strategy is termed „drug repurposing" (or drug re-profiling), during which new pharmacological uses are identified for drugs already approved, outside of their original indications (Pushpakom et al. 2019). As the physicochemical, pharmacokinetic and toxicological profile of these compounds have already been established, the initial stages of the drug authorization process (Phase I–II clinical trials) may be avoided, leading to substantial monetary benefits for the pharmaceutical companies; if this new indication of the tested compound is appropriate, pharmaceutical companies may once again expect financial returns for their investments (Miró-Canturri et al. 2019; Paul et al. 2010; Pushpakom et al. 2019; Soo et al. 2017). Drug re-profiling is also an emerging strategy in antimicrobial chemotherapy: some of these compounds have antibacterial properties themselves, while others have secondary mechanisms of action (some of which are unknown as of now). These mechanisms may include: bacteriostatic properties, inhibition of bacterial cell-cell communication, modulation of virulence factor-expression, biofilm-inhibition and so on (Miró-Canturri et al. 2019; Paul et al. 2010; Pushpakom et al. 2019; Soo et al. 2017; Yang et al. 2009) However, there are still significant gaps in the knowledge in the field of drug repurposing for antimicrobial purposes.

The aim of our present study was to assess the adjuvant properties of several existing and widely-used pharmacological agents against bacteria in combination with reference antibiotics, in an *in vitro* study.

Materials and methods

Culture media

The following culture media were used during our experiments: cation-adjusted Mueller-Hinton broth (Bio-Rad, Hercules, CA, USA), Luria–Bertani broth (Bio-Rad, Hercules, CA, USA), 5% sheep blood agar (bioMérieux, Marcy-l'Étoile, France) and eosine-methylene blue agar (bioMérieux, Marcy-l'Étoile, France).

Bacterial strains

Staphylococcus aureus ATCC 25923 and *S. epidermidis* ATCC 12228 were used as representative Gram-positive strains, while *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were selected as representative Gram-negative strains for our experiments. For shorter time periods (<1 month), the bacterial strains were maintained on blood agar and eosine-methylene blue agar plates (for Gram-negatives) with continuous passage. For longer periods, the strains were kept in a -80 °C freezer, in a 1:4 mixture of 85% glycerol and liquid Luria-Bertani medium.

Antibiotics and non-antibiotic compounds

Ciprofloxacin and gentamicin (Sigma-Aldrich, St. Louis, MI, USA; will be listed as SA in the subsequent text) were selected as antibiotic controls for our studies. Twenty (n = 20) pharmacological agents, encompassing drug with different chemical structures and mechanisms of action were tested during our experiments: acetaminophen (SA), amantadine (SA), acyclovir (Teva Pharmaceuticals, Petah Tikva, Israel; will be listed as TPh in the subsequent text), atorvastatin (SA), azelastine (SA), celecoxib (Pfizer Hungary, Budapest, Hungary), cetirizine (SA), clotrimazole (TPh), diclofenac-epolamine (SA), enalapril-maleate (SA), ivermectin (SA), lidocaine (SA), mebendazole (SA), metformin (SA), metoprolol-succinate (SA), prazozine (SA), sitagliptine (SA), terbinafine (GlaxoSmithKline, Brentford, UK), valsartan (SA) and xylomethazoline (SA). The compounds were chosen on the basis of being available as over-the-counter (OTC) medication or being frequently prescribed for common chronic conditions as hypertension or diabetes mellitus. Pharmaceutical compounds were dissolved in phosphate-buffered saline (PBS), except for atorvastatin, which was dissolved in dimethyl sulfoxide (DMSO). All solutions were prepared on the day of the assay. The concentration of DMSO was below 1 V/V% in all experiments.

Antibacterial activity of non-antibiotic compounds, MIC determination

The minimum inhibitory concentrations (MICs) of the tested compounds were determined using the standard broth microdilution method, based on the recommenda-

Table 1 Minimum inhibitory concentrations (MICs) of the tested pharmaceutical compounds on reference bacterial strains.

	Minimal inhibitory concentrations (μg/mL)			
Compounds	*S. aureus* ATCC 25923	*S. epidermidis* ATCC 12228	*E. coli* ATCC 25922	*K. pneumoniae* ATCC 700603
Acetaminophen	>250	>250	>250	>250
Amantadine	>250	>250	>250	>250
Acyclovir	>250	>250	>250	>250
Atorvastatin	125	125	250	>250
Azelastine	>250	>250	>250	>250
Celecoxib	15.6	31.2	>250	>250
Cetirizine	>250	>250	>250	>250
Clotrimazole	125	62.5	>250	>250
Diclofenac-epolamine	250	250	>250	>250
Enalapril-maleate	>250	>250	>250	>250
Ivermectin	31.2	125	>250	>250
Lidocaine	250	250	250	250
Mebendazole	62.5	125	62.5	250
Metformin	>250	>250	>250	>250
Metoprolol-succinate	>250	>250	>250	>250
Prazozine	>250	>250	>250	>250
Sitagliptine	>250	>250	>250	>250
Terbinafine	250	125	>250	>250
Valsartan	>250	>250	>250	>250
Xylomethazoline	>250	>250	>250	>250

tions of the Clinical and Laboratory Standards Institute (CLSI; M07-A10). The experiments were performed in 96-well polystyrene microtiter plates, using cation-adjusted Mueller–Hinton broth. The tested concentrations of the compounds were ranging between 1.95-250 μg/mL, the two-fold serial dilutions of the tested compounds were made starting in the third row of the microtiter plates. During the experiments with *S. aureus* ATCC 25922 and *S. epidermidis* ATCC 12228, the Mueller-Hinton broth was supplemented by 2% NaCl, as based on CLSI protocols. The plates were incubated at 37 °C in an air thermostat. The MIC values of the tested compounds were recorded after 16-18 h of incubation; the interpretation of the results was performed visually. All experiments were performed in triplicate.

MIC reduction assay

To ascertain the effect of the tested compounds on the MICs of standard antibiotics (i.e. ciprofloxacin and gentamicin), a MIC reduction assay was performed (Sarker et al. 2007). The assay was performed in a 96-well microtiter plate, using cation-adjusted Mueller-Hinton broth. The setup of the plates was the following: in rows A-D of the plate, serial dilutions were made for the reference antibiotic, in rows E-H the same serial dilutions were performed for the reference antibiotic with the addition of the non-antibiotic compounds in a constant concentration as adjuvants (MIC/4 in cases where MIC was ≤ 250 μg/mL and 125 μg/mL where MICs were higher than 250 μg/mL) in all the wells, except for medium control and cell control wells (Sarker et al. 2007). The inoculation of the plates and the incubation was performed according to a standard broth microdilution method, described previously. The modified MICs (compared to the MICs of the antibiotics alone) were determined visually, as the concentration, where no visible growth of bacteria could be observed. All experiments were carried out in triplicate.

Results

Antibacterial activity of pharmaceutical compounds

The MICs observed for the non-antibiotic pharmaceutical compounds is presented in Table 1. Eight tested compounds (namely atorvastatin, celecoxib, clotrimazole, diclofenac-epolamine, ivermectin, lidocaine, mebendazole and terbinafine) showed antibacterial activities in the tested concentration range, while compounds with MICs >250 μg/mL were not considered to be active.

MIC reduction assays

The results of the MIC reduction assays for Gram-positive

Table 2 Results of the MIC reduction assays on Gram-positive bacterial strains using ciprofloxacin and gentamicin as reference antibiotics.

	Minimal inhibitory concentrations (μg/mL)			
Compounds	*S. aureus* ATCC 25923		*S. epidermidis* ATCC 12228	
MICs of reference antibiotics:	Ciprofloxacin: 0.12 μg/mL	Gentamicin: 0.12 μg/mL	Ciprofloxacin: 0.12 μg/mL	Gentamicin: 0.06 μg/mL
Acetaminophen	0.12	0.12	0.12	0.06
Amantadine	0.12	0.12	0.12	0.06
Acyclovir	0.12	0.12	0.12	0.06
Atorvastatin	**0.015**	**0.06**	**0.0075**	**0.03**
Azelastine	**0.06**	0.12	0.12	0.06
Celecoxib	**0.015**	0.12	**0.03**	0.06
Cetirizine	0.12	0.12	0.12	0.06
Clotrimazole	**0.06**	**0.06**	**0.06**	**0.03**
Diclofenac-epolamine	**0.06**	0.12	**0.03**	0.06
Enalapril-maleate	0.12	0.12	0.12	0.06
Ivermectin	**0.03**	0.12	**0.03**	0.06
Lidocaine	**0.06**	**0.06**	**0.06**	0.06
Mebendazole	**0.03**	0.12	0.12	0.06
Metformin	0.12	0.12	0.12	0.06
Metoprolol-succinate	0.12	0.12	0.12	0.06
Prazozine	0.12	0.12	0.12	0.06
Sitagliptine	0.12	0.12	0.12	0.06
Terbinafine	**0.015**	0.12	**0.03**	0.06
Valsartan	**0.06**	**0.06**	**0.03**	**0.03**
Xylomethazoline	0.12	0.12	0.12	0.06

Results in boldface represent cases when the MICs have decreased due to the effect of the adjuvants.

bacteria are presented in Table 2., while results for Gram-negative bacteria are shown in Table 3. Overall, the tested non-antibiotics were the most potent adjuvants against Gram-positive bacteria and they enhanced the antibacterial activity (i.e. they reduced the MICs) or ciprofloxacin to the highest extent (reducing the MICs 50-93.25% or 2-5-fold), while having modest effects on Gram-negative bacteria (*E. coli* and *K. pneumoniae*) and on the MICs of gentamicin. Interestingly, azelastine and valsartan on Gram-positive bacteria, while cetirizine, enalapril, valsartan and xylomethazoline on Gram-negative bacteria had MIC-reducing effects, without having any intrinsic antibacterial properties themselves (see Table 1.).

Discussion

Infections caused by MDR bacteria are associated with an increased mortality rate and decreased quality of life in the affected patients worldwide (Falagas et al. 2008). Since the 2000s, the development of novel antibiotics has been shown to keep up with the development in the levels of bacterial resistance (Falagas et al. 2008). Combination therapy with non-antibiotic compounds may provide a straightforward, attractive and financially reasonable drug development avenue (Medina and Pieper 2016; Lyddiard et al. 2016; Spellberg 2014). Several reports have been published in the literature on the antibacterial properties of non-antibiotic drugs; however, the systematic screening of drugs for such purposes have not yet been performed (Kruszewska et al. 2002; Lagadinou et al. 2020). Doxorubicin and bleomycin are antitumor agents (frequently termed as "anticancer antibiotics") show antibacterial properties on a variety of bacterial strains: the proposed mechanism of action is related to their intercalation into bacterial DNA (similar to the mechanism of the fluoroquinolones) and the generation of oxidative free radicals in the presence of Fe^{2+}-ions (which are indispensable for the biochemical pathways of bacteria) (Kruszewska et al. 2002; Lagadinou et al. 2020; Soo et al. 2017). Several antipsychotic drugs (e.g., phenothiazine, thioridazine) have also been described as DNA-intercalators; in addition, their efflux pump-inhibitory properties were also experimentally verified on many bacterial strains (Amaral et al., 2004). The adjuvant properties of non-steroidal anti-inflammatory drugs (NSAIDs) in bacterial infections have been supported in the clinical practice by empirical evidence, while laboratory studies have also

Table 3 Results of the MIC reduction assays on Gram-negative bacterial strains using ciprofloxacin and gentamicin as reference antibiotics.

	Minimal inhibitory concentrations (μg/mL)			
Compounds	*E. coli* ATCC 25922		*K. pneumoniae* ATCC 700603	
MICs of reference antibiotics:	Ciprofloxacin: 0.004 μg/mL	Gentamicin: 0.5 μg/mL	Ciprofloxacin: 0.12 μg/mL	Gentamicin: 0.05 μg/mL
Acetaminophen	0.004	0.5	0.12	0.5
Amantadine	0.004	0.5	0.12	0.5
Acyclovir	0.004	0.5	0.12	0.5
Atorvastatin	0.004	0.5	0.12	0.5
Azelastine	0.004	0.5	0.12	0.5
Celecoxib	**0.001**	0.5	**0.03**	0.5
Cetirizine	**0.001**	**0.25**	**0.6**	0.5
Clotrimazole	0.004	0.5	0.12	0.5
Diclofenac-epolamine	**0.002**	0.5	**0.6**	0.5
Enalapril-maleate	**0.002**	0.5	0.12	0.5
Ivermectin	0.004	0.5	0.12	0.5
Lidocaine	**0.001**	0.5	**0.03**	0.5
Mebendazole	0.004	0.5	0.12	0.5
Metformin	0.004	0.5	0.12	0.5
Metoprolol-succinate	0.004	0.5	0.12	0.5
Prazozine	0.004	0.5	0.12	0.5
Sitagliptine	0.004	0.5	0.12	0.5
Terbinafine	0.004	0.5	0.12	0.5
Valsartan	**0.001**	**0.125**	**0.6**	0.5
Xylomethazoline	**0.002**	0.5	0.12	0.5

Results in boldface represent cases when the MICs have decreased due to the effect of the adjuvants.

provided results that some NSAIDs (e.g., acetaminophen, acetyl-salicylic acid, ibuprofen, indomethacin, metamizol-sodium, etoricoxib) and local anesthetics (e.g., lidocaine) may have mechanisms enhancing the effects of antibiotics *in vivo* (Al-Bakri et al. 2009; Chan et al. 2017; D'Angelo et al. 2018; Johnson et al. 2008; Ogundeji et al. 2016; Thangamani et al. 2015). These mechanisms may include inhibition of biofilm-formation, adherence, reduction of motility and the modulating the release of antibiotics by leukocytes (Al-Bakri et al. 2009; Chan et al. 2017; D'Angelo et al. 2018; Johnson et al. 2008; Ogundeji et al. 2016; Thangamani et al. 2015). Allopurinol (a gout medication) increased the potency of anti-tuberculosis medications against *Mycobacterium tuberculosis* (Naftalin et al. 2017). The antibacterial activity of azole antifungals and ivermectin against Gram-positive bacteria only was previously described (Ghannoum and Rice 1999; Ashraf et al. 2018). The exact mechanism of action is not well-defined, but it is probably associated with affecting the binding to the terminal D-alanyl-D-alanine of the pentapeptide peptidoglycan precursors in the cell wall (Ghannoum and Rice 1999; Ashraf et al. 2018). The mechanism of statins (including simvastatin and atorvastatin among others) regarding their antibacterial potency also needs further studies, however, it has been suggested that they interfere with the mevalonate pathway, limiting the synthesis of the major lipid constituents of cell membrane microdomains (Ko et al. 2017). Apart from drugs, some publications also reported on the adjuvant properties of vitamins, enhancing the bactericidal activity of antibiotics; these publications highlight the role of high-dose of lipid soluble vitamins (ADEK) and Vitamin C (Andrade et al. 2014; Kwiencinska-Piróg et al. 2019).

Conclusions

The aim of our present study was to assess a selection of non-antibiotic pharmaceutical compounds – sourced from diverse clinical indications and molecular characteristics – as antibiotic adjuvants. Currently, there are around 6000-9000 drug compounds marketed for human therapeutic purposes; these agents may be considered as potential combination agents with reference antibiotics, to potentiate their antibacterial properties in clinical situations. The pharmacokinetic parameters and *in vivo* tolerability of these compounds have already been described; thus, these compounds are already one

step closer into their clinical utilization. The highlights of the study include the study of twenty pharmaceutical compounds that are frequently used by patients. Eight tested compounds showed antibacterial activity on the tested bacterial strains and several agents presented with various degrees of adjuvant (MIC-reducing) properties. Further experiments involving the screening of additional pharmaceutical compounds for their secondary antibacterial and adjuvant properties is definitely warranted.

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