Non-antibiotic compounds affecting the growth of urinary pathogens during urine culture: a preliminary *in vitro* study

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Introduction: *Urine samples are one of the most frequently submitted samples for culture to clinical microbiology laboratories, exceeding the number of most of the other clinical sample types. Various non-antibiotic pharmaceutical compounds may have inhibitory properties on bacteria, as many of these agents accumlate in/eliminated through urine.*
Aims: *The aim of our present study is to screen various non-antibiotic group pharmacological agents in vitro for their potential to augment the viability of pathogenic bacteria in urine samples.*
Methods: *Sixty (n=60) pharmacological agents were tested during our experiments. Bacillus subtilis ATCC 6633, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 700603 (ESBL-producing) and Staphylococcus aureus ATCC 29213 were the bacterial strains utilized in this study. Detection of inhibitory activity among the tested compounds was performed on Mueller-Hinton plates, using disk diffusion method.*
Results: *Nineteen (n=19) compounds presented with various levels of inhibitory activity on the tested bacterial strains (four compounds for K. pneumoniae, seven compounds on E. coli and sixteen compounds on S. aureus). The compounds showed the highest levels of inhibitory activity on B. subtilis ATCC 6633, which is one of the main bacterial strains used for the screening of the 'intrinsic' antibacterial activity of urine.*
Conclusion: *During urinalysis, all possible confounding variables must be taken into consideration, which may distort the culture results of routine laboratories. Our results suggest that further experiments, involving additional pharmacological agents is warranted, to establish the full extent of their influence on the appropriate culture of urine samples.*

Keywords: *urinary tract infections; urinalysis; intrinsic antibacterial activity; non-antibiotics; antimicrobials; drug repurosing; disk diffusion; Bacillus subtilis*

1. Introduction

Urinary tract infections (UTIs) are one of the most common infectious pathologies worldwide (following lower respiratory tract infections and gastrointestinal infections) [1,2]. From the standpoint of public health, UTIs represent an important factor or morbidity and mortality, affecting both patients in primary care and tertiary care settings [3]. In fact, according to some estimates, around 50-60% of women in the age range of 20–40 years experience a UTI at least once during their lifetime, while nosocomial UTIs may represent 25–50% of hospital-acquired infections overall [4]. The diagnosis and management of UTIs, and the corresponding lost working days associated with these infections also have a significant economic consequence, estimated to be around 3-5 billion US dollars annually [5,6]. Uncomplicated UTIs are principally associated with members of the intestinal flora, with *Escherichia coli* representing 50-90% of these etiologies [7,8]; the spectrum of pathogens assicoated with nosocomial infections is more diverse, including non-fermenting Gram-negative bacteria, Gram-positive cocci (*Staphylococcus aureus*, *S. saphrophyticus*, *Enterococcus* spp.) and *Candida* spp [9-11]. UTIs are associated with a variety of clinical signs and symptoms, including the burning sensation in the genitourinary region, strong and persistent urge to urinate, small volume of voided urine, urinary incontinence, pelvic pain, fever and nausea/vomiting [12]. Additionally, the color and consistency of the voided urine may be also subject to changes (cludy, red, bright pink, bloody, and foul-smelling urine) [12,13].

Urine samples (more commonly clean-catch/midstream and catheter-specimen urine) are one of the most frequently submitted samples for culture to the clinical microbiology laboratories, exceeding the number of most of the other clinical

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sample types [14]. Clean-catch urine samples are an inexpensive and non-invasive without the risk of complications; although contamination of the sample with the normal flora or the distal urethrea is a risk, the appropriate instruction of patients regarding hygienic considerations and sample collection is usually adequate for appropriate samples to be attained [15]. Nevertheless, collection of urine by using a single catherer is a more appropriate method to use to avoid contamination in hospitalized patients [1,2,15]. Bacteriological culture of urine samples on non-selective or chromogenic media (frequently coupled with the use of nitrite and leukocyte-esterase tests or a hemocytometer) is the gold standard method in the etiological diagnosis of UTIs. The interpretation of culture results (usually $\geq 10^5$ colony forming units/mL corresponding to singificant bacteriuria) from urine samples provide little or no challenge to clinical microbiologists [16]. Based on data from the literature, 50-70% of urine cultues are culture-negative, while out of the positive urine samples, 40-50% of isolated bacteria are relevant urinary pathogens [17]. Sample procurement, time elapsed before sample processing and expertise of the staff are all relevant factors in establishing the etiology of UTIs. However, some additional factors may influcence the results of succesful interpretation of urine cultues. It it well-known that microbiological sampling should preferably be carried out before the administration of antibiotics, as these drugs may lead to false negative results (inhibiting or significantly reducing bacterial growth), misleading clinicians and microbiologists [18]. To screen for this, routine microbiology laboratories often perform ancillary tests with pan-susceptible bacterial strains (e.g., *Bacillus* spp., *E. coli*) to assess the intrinsic antibacterial activity of the urine samples [19]. If these tests prove to be positive, clinical microbiologists may observe different rules during interpretation of culture results.

Nevertheless, there is increasing evidence that various non-antibiotic pharmaceutical compounds may also have inhibitory properties on bacteria [20]; as a part of drug repurposing advances, several drugs have also been screened for their antimicrobial properties [21]. In addition, the pharmacokinetic properties of these drugs should also be taken into consideration, as many of these agents accumlate in/eliminated through urine, thus, they may possess the potency to adversely affect the growth of uropathogenic bacteria [22]. Therefore, the aim of our present study is to screen various non-antibiotic group pharmacological agents *in vitro* for their potential to augment the viability of pathogenic bacteria in urine samples or their growth on culture media during urinalysis.

2. Materials and Methods

2.1. Chemicals

Sixty (n=60) pharmacological agents, encompassing a wide variety of different chemical structures and mechanisms of action were tested during our experiments: acetylsalicylic acid (Sigma-Aldrich; Budapest, Hungary; will be listed as SA in the subsequent text), acetaminophen (SA), acetyl-cysteine (Teva Pharmaceuticals; Petah Tikva, Israel; will be listed as TPh in the subsequent text), acyclovir (TPh), allopurinole (SA), amantadine (SA), ambroxol (TPh), atorvastatin (SA), atracurium (SA), azelastine (SA), bleomycin (TPh), cisplatin (TPh), celecoxib (Pfizer Hungary Ltd.; Budapest, Hungary), cetirizine (SA), chlorpromazine (SA), chloroxazone (SA), cidofovir (SA), clotrimazole (TPh), cyclophosphamide (Baxter; Deerfield, IL, United States), diclofenac (SA), doxorubicin (TPh), enalapril maleate (SA), etodolac (SA), famotidine (SA), fluconazole (SA), fluoxetine (SA), gemcitabine (TPh), guaifenesin (SA), indomethacin (Sanofi; Paris, France; will be listed as SP in the subsequent text), imipramine (SA), ivermectin (SA), metamizole-sodium (SF), mebendazole (Richter Pharmaceuticals; Budapest, Hungary; will be listed as RPh in the subsequent text), lidocaine (SA), metoprolol succinate (SA), paclitaxel (TPh), prazozin (SA), metformin (SA), methotrexate (Ebewe Pharma, Unterach am Attersee, Austria), prilocaine (SA), promethazine (SA), risperidone (SA), simvastatin (SA), sitagliptine (SA), suxamethonium (SA), terbinafine (GlaxoSmith-Kline Hungary Ltd., Budapest, Hungary), thioridazine (SA), topotecan (SA), valsartan (SA), verapamil (TPh), vincristine (TPh), xylomethazoline (SA), Vitamin B_1 (EGIS Pharmaceuticals; Budapest, Hungary; will be listed as EGIS in the subsequent text), Vitamin B_6 (EGIS), Vitamin B_{12} (RPh), Vitamin C (SA), Vitamin D (EGIS), Vitamin E (SA), Vitamin K (SA) and 5-fluorouracil (TPh). The compounds were chosen on a basis of being substrates of the organic cation transporter-2 (OCT2/SLC22A2), organic anion transporters 1 and/or 3 (OAT1/SCL22A6 and OAT3/SCL22A8) and multi-antimicrobial extrusion protein (MATE), which are all relevant transporters in

the renal elimination of various pharmacological agents [23]. The list of relevant substrates was acquired from the DrugBank database (https://www.drugbank.ca/).

Pharmaceutical compounds were dissolved in phosphate-buffered saline, with the exception of simvastatin and atorvastatin, which were dissolved in dimethyl sulfoxide (DMSO), in addition to Vitamin D and Vitamin K, which were dissolved in acetone and 70% ethanol, respectively. The final concentration of the tested compounds was set at 100 μg/mL in the experiments.

2.2. Bacterial strains

The following bacterial strains were used during our growth inhibition experiments: *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603 (ESBL-producing) and *Staphylococcus aureus* ATCC 29213.

2.3. Culture media, paper disks

Bacterial strains were maintained on blood agar and eosine methylene blue plates (bioMérieux, Marcy-l'Étoile, France). Inhibitory activity of the tested compounds was investigated on Mueller-Hinton agar plates (bioMérieux, Marcy-l'Étoile, France).

Filter paper disks (7.0 mm in diameter, Whatman 3MM) were impregnated with the solutions of the tested compounds. Ciprofloxacin (5 μg), meropenem (10 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg) disks (Liofilchem, Abruzzo, Italy) were used in the control experiments.

2.4. Inhibitory activity of non-antibiotic drugs

Detection of inhibitory activity among the tested compounds was performed on MHA plates, containing *B. subtilis* ATCC 6633 spores [22,24,25]. A maximum of 6 sterile filter paper discs (impregnated with 10 μL of the solutions of the solutions of different the tested compounds) were placed on MHA, containing a *B. subtilis* spore suspension (250 μl per 1 liters). Control strains (*S. aureus*, *E. coli* and *K. pneumoniae*) were plated on MHA agar conventionally, and the sterile filter paper discs were placed on the inoculated plates. The plates were incubated at 37 °C in an air thermostat. The inhibitory activity of the tested compounds was assessed semi-quantitatively; the zone of inhibition around the disks impregnated with the solutions of the tested compounds were recorded after 16–18 h of incubation, using a caliper (expressed as milimeters ± standard deviation [SD]). Any measureable zone of inhibition was considered as positive [22,24,25]. DMSO (at 2 V/V% concentration) was used as a negative control for the tested compounds, while ciprofloxacin, meropenem and trimethoprim/sulfamethoxazole disks were used as positive controls. All experiments were performed in triplicate.

3. Results

Out of the 60 tested pharmacological agents, nineteen (n=19) compounds presented with various levels of inhibitory activity on the tested bacterial strains. The results of our disk diffusion inhibitory experiments are presented in Table I. Out of the nineteen compounds, four compounds (atracurium, doxorubicin, lidocaine, thioridazine) showed measurable inhibition zones on *K. pneumoniae* ATCC 700603 (ranging between 2-6 mm), while seven compounds (atracurium, celecoxib, chlorpromazine, doxorubicin, imipramine, lidocaine, thioridazine) showed inhibitory activity on *E. coli* ATCC 25922 (with zone diameters ranging between 1-7 mm*). S. aureus* ATCC 29213 was more susceptible to the inhibitory activity of the tested drugs (zone diameters ranging between 4-14 mm; for 16 out of the 19 compounds), with the exception of allopurinole, methotrexate and verapamil). The compounds showed the highest levels of inhibitory activity on *B. subtilis* ATCC 6633, which is one of the main bacterial strains used for the screening of the 'intrinsic' antibacterial activity of urine; with zone diameters ranging between 4 mm (allopurinole) and 22 mm (thioridazine). All tested reference antibiotics showed zone diameters for the respective bacterial strains, which corresponded to the 'susceptible' therapeutic category (based on EUCAST v. 9.0 breakpoints). 2 V/V% DMSO did not show any inhibitory activity during the experiments.

4. Discussion

UTIs are a major publich health and economic burden to healthcare infrastructres worldwide, therefore the correct determination of the etiological agents in these infections in of utmost importance [1-3, 5, 11, 25, 26]. During urinalysis, all possible confounding variables must be taken into consideration, which may distort the culture re-

Table I *Inhibitory activity of tested pharmaceutical compounds (results expressed as mm ± SD)*

	Bacillus subtilis **ATCC 6633**	***Escherichia coli*** **ATCC 25922**	***Klebsiella pneumoniae*** **ATCC 700603**	***Staphylococcus aureus*** **ATCC 29213**
Allopurinole	4 ± 1	Ø	Ø	Ø
Atorvastatin	11± 2	Ø	Ø	8 ± 2
Atracurium	14 ± 2	5 ± 1	3 ± 1	6 ± 1
Bleomycin	16 ± 2	Ø	Ø	8 ± 3
Celecoxib	20 ± 3	1 ± 1	Ø	14 ± 2
Chlorpromazine	17 ± 3	3 ± 1	Ø	10 ± 2
Clotrimazole	15 ± 2	Ø	Ø	5 ± 2
Doxorubicin	18 ± 3	5 ± 2	5 ± 1	8 ± 2
Etodolac	15 ± 3	Ø	Ø	7 ± 1
Fluconazole	17 ± 1	Ø	Ø	7 ± 2
Imipramine	9 ± 2	3 ± 3	Ø	4 ± 2
Ivermectin	14 ± 3	Ø	Ø	8 ± 3
Lidocaine	17 ± 4	7 ± 2	6 ± 1	10 ± 3
Mebendazole	16 ± 1	Ø	Ø	12 ± 2
Methotrexate	10 ± 2	Ø	Ø	Ø
Promethazine	7 ± 2	Ø	Ø	6 ± 3
Simvastatin	13 ± 2	Ø	Ø	10 ± 2
Thioridazine	22 ± 4	5 ± 1	2 ± 1	9 ± 3
Verapamil	6 ± 3	Ø	Ø	Ø
Ciprofloxacin (5 μg)	24 ± 3	27 ± 3	26 ± 2	26 ± 2
Meropenem (10 μg)	29 ± 2	24 ± 1	23 ± 1	24 ± 1
Trimethoprim/sulfamethoxazole (1.25/23.75 μg)	16 ± 3	19 ± 1	18 ± 2	16 ± 2

Ø: no inhibition zones were observed

sults of routine laboratories. These may include issues during sample procurement and time elapsed before sample has been processed (i.e. the pre-analytical phase), however, troubleshooting must also encompass steps in the analytical phase [27]. The chemical composition of urine clearly affects the viability and species-composition of bacteria, for example, if the pH of the urine shifts in either directions, it may inhibit or potentiate the replication of several microorganisms [26,27]. Many natural compounds and constituents of our diet have well-known antibacterial properies (e.g., ajoene [28], betulinic acid [29], cranberry juice [30], curcumin [31], essential oils [32], horse raddish [33], pepper [34], resveratrol [35] and zeaxantin [36]), which may influence bacterial viability in urine. Nevertheless, the relevance of non-antibiotic compounds in this regard must not be underestimated [20,21,37]; this is especially true in case of older patients, whom many drugs are simulatenously prescribed [38]. In our study, nineteen out of the sixty tested pharmacological agents presented with growth inhibitory properties on the tested bacterial strains. With the inclusion of *S. aureus*, *E. coli* and *K. pneumoniae* in the study, we aimed to assess the relevance of these drugs in decreasing the viability of pathogenic bacteria in urine; in contrast, the *B. subtilis* strain is predominantly used to provide information on the antibacterial activity of the urine sample itself. While 4-16 compounds (depending on the bacterial strain) showed growth inhibitory activity on the reference strain, n=19 drugs inhibited the growth of *B. subtilis* in the disk diffusion tests to various extents. This experiental result may point out that in addition to antibiotics, non-pharmacological agents may also be responsible to „positive" tests, when assessing the antibacterial activity of the urine samples received, depending on the concentration, in which they are available in the

urine [22]. Similarly to our results, the potential antibacterial activity of azole antifungal agents [39], antracyclines [40], phenothiazines [41], local and general anesthetics [42], peripherially acting muscle relaxants [43], non-steroidal anti-inflammatory drugs [44] and statins [45] were already demonstrated by studies in different settings. However, other studies also highlighted the antibacterial properties of acetyl-salicylic acid [46], allopurinole [47], various cardio-vascular medications [48], and several vitamins (A, C, D and K) [49-52]; this was not demonstrated in our *in vitro* settings.

5. Conclusions

In conclusion, the aim of our present study was produce *in vitro* data on the possible role of non-antibiotic pharmacological agents, as inhibitors of growth during urinalysis, i.e. the culture of urine samples on bacteriological media, if a UTI is suspected. Our results show that a wide variety of structurally unrelated drugs may have the potential to inhibit the growth of urinary pathogens, or *B. subtilis*, a commonly used microorganism in ancillary tests. Although the methodology used during our experients (disk diffusion) offers only preliminary, semi-quantitative results and the experiments were carried out in a select group of bacteria, our results suggest that further experiments, involving additional pharmacological agents is warranted, to establish the full extent of their influence on the appropriate culture of urine samples.

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Conflicts of interest

The author declares no conflict of interest, monetary or otherwise.

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