Fitoterapia 129 (2018) 237–240

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Treating urinary tract infections due to MDR *E. coli* with Isothiocyanates – a phytotherapeutic alternative to antibiotics?

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ARTICLE INFO

Keywords:
Isothiocyanates
Urinary tract infection
Multidrug resistance
Uropathogenic *E. coli*

ABSTRACT

Background: Multidrug-resistant (MDR) bacteria are increasingly causing urinary tract infections (UTI), which has been linked to frequent use of antibiotics. Alternative treatment regimens are urgently needed and natural isothiocyanates (ITC) may represent one. ITCs are natural plant products found in nasturtium (*Tropaeoli majoris herba*) and horseradish (*Armoraciae rusticanae radix*).
Purpose: The objectives were to (1) assess the antimicrobial effects of nature-identical ITCs for UTI treatment caused by uropathogenic *E. coli* (UPEC), (2) to evaluate a potential influence of antimicrobial resistance on ITC susceptibility, and (3) to test whether ITCs affect UPEC penetration into human uroepithelial cells.
Methods: We tested 217 clinical UPEC isolates, 54.5% of which were classified as MDR, for susceptibility against ITCs. ITC susceptibility testing was performed by broth dilution using a mixture of three synthetic ITCs. Internalization was tested using human T-24 bladder carcinoma cells in an internalization assay co-incubated with UPEC ($n = 5$) and ITCs.
Results: The mean minimal inhibitory concentration (MIC) 90 was 0.17 mg/ml, showing very high susceptibility against ITCs. Interestingly, MDR *E. coli* were significantly less susceptible than non-MDR strains ($p = .01$). Internalization of UPEC was decreased by 31.9% in the mean when treated with ITCs. Overall, ITCs exerted a strong antimicrobial activity against clinical UPEC isolates and reduced internalization into uroepithelial cells.
Conclusion: ITCs might present a promising treatment alternative for UTIs, expressing both high antimicrobial activity as well as blocking the pathogenic process of human cell penetration by UPEC. Clinical studies, however, are needed to confirm activity of ITCs in UTIs *in vivo*.

1. Introduction

Urinary tract infections (UTIs) are common bacterial infections in out - as well as inpatient care and the second most common reason to prescribe antibiotics [1], often prematurely. Almost every third woman will be diagnosed with a UTI, requiring antibiotic therapy before the age of 25 [2]. Uropathogenic *Escherichia coli (UPEC),* the most frequently isolated species in UTIs [3], are able to survive antibiotic treatment through internalization and cause relapses. An Italian study estimated that the costs associated with a single episode of UTI amount to 239 Euros [4]. Taking the high frequency of UTIs into account, the economic impact is considerable. An estimated one billion US dollars is spent on the management of UTIs annually [5].

Besides economic factors, the increasing spread of antibiotic resistance is of growing concern [6]. Large epidemiological studies showed high resistance rates among pathogens causing UTIs [3, 7]. The Antimicrobial Resistance Epidemiological Survey on Cystitis, for instance, rated > 10% of their *E. coli* isolates as multidrug-resistant (MDR) [3]. Hence, finding alternative treatment and relapse prevention strategies is of utmost importance [8].

Nature-identical isothiocyanates (ITCs), set free by enzymic hydrolysis of glucosinolates found in Brassicaceae vegetables, are considered such an alternative [9, 10] as they might exhibit antimicrobial activity [11, 12]. ITCs derive from nasturtium (*Tropaeoli majoris herba*) and

Abbreviations: CFU, colony-forming unit; ESBL, Extended-Spectrum-Betalactamase; ITC, natural isothiocyanates; MDR, Multidrug-resistant; MIC, minimal inhibitory concentration; UPEC, uropathogenic *E. coli*; UTI, urinary tract infections

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https://doi.org/10.1016/j.fitote.2018.07.012
Received 21 June 2018; Received in revised form 16 July 2018; Accepted 18 July 2018
Available online 19 July 2018

horseradish (*Armoraciae rusticanae radix*) and are therefore found in nature [11, 12]. The objective of this study was to assess the clinical potential of nature-identical ITCs for the treatment of UTIs. We assessed its antimicrobial efficacy and its capability to reduce internalization of UPEC. Therefore, we tested the ITC susceptibility of *E. coli* isolated from clinical patients with signs and symptoms of UTI. Furthermore, we compared MDR and non-MDR strains to detect possible differences in their susceptibility to ITC. We then assessed the internalization behavior of five different *E. coli* strains into cultivated human uroepithelial cells with and without ITC present.

2. Material and methods

2.1. Antimicrobial susceptibility testing

We isolated 217 *E. coli* strains from UTI patients of the Freiburg University Hospital. UTI with *E. coli* was defined according to German laboratory standards (M. Hauch et al. Microbiological-infectious disease diagnostic quality standards [MiQ]). Briefly, *E. coli* UTI was considered by isolation in midstream urine of: (1) pure culture of *E. coli* in 10^3–10^4 CFU or (2) isolation of *E. coli* in > 10^4 CFU or (3) isolation of *E. coli* pure culture in puncture urine with any CFU. Additionally, we included five reference strains (*E. coli* ATCC 11,229, 11,775, 12,155, 25,922, 9,637) for standard antimicrobial susceptibility testing using the VITEK2 (bioMérieux, France) system. Interpretation of results was done according to EUCAST clinical breakpoints. Strains were classified as MDR or non-MDR as previously described [13]. Briefly, strains were classified as MDR when they were ESBL positive (expressing penicillin and cephalosporin resistance) and quinolone resistant.

2.2. ITC susceptibility testing

We used allyl - ITC (Sigma – Aldrich with a purity of 95% and Merck KGaA with a purity of at least 94%), benzyl - ITC (Sigma – Aldrich with a purity of 98% and Fluka Analytical with a purity of at least 97%) and 2 - phenyl – ethyl - ITC (Fluka Analytical with a purity of at least 95%). A mixture of 38% (v / v) allyl - ITC, 50% benzyl - ITC and 12% 2 – phenyl – ethyl - ITC was obtained to reflect the proportions of active agents in a phytotherapeutic drug (Angocin® Anti-Infekt N, Repha GmbH) available in Germany [14]. To dilute this lipophylic mixture, 8% (v / v) polysorbate 80 (Merck Schuchardt OHG) served as a solvent.

One ml of a stock solution, containing 2% (v / v) of the pure ITC mixture, was used in a serial dilution. Subsequently, 0.5 ml double concentrated Mueller Hinton broth (Merck KGaA), containing 10 ^ 6 colony - forming units per ml (CFU / ml), was added to each tube. Resulting ITC concentrations ranged from 0.02 to 10.80 mg / ml.

To rule out contamination, a mixture of polysorbate and Mueller Hinton broth was used as a negative control. Additionally, the polysorbate was mixed in a one – to - one ratio with the bacterial solution to prove growth - enhancing test conditions. The quality of the bacterial solution used was also tested. It was, therefore, diluted to a concentration of 10 ^ 4 CFU / ml. A sample of 20 µl was then plated on Mueller Hinton agar (Merck KGaA). All samples were incubated at 36 °C for 24 h. The lowest ITC concentration with no visible bacterial growth indicated the minimal inhibitory concentration (MIC).

2.3. UPEC internalization

2.3.1. Cell lines and bacteria

Human T-24 bladder carcinoma cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ No. ACC376) were used as human test cells. T-24 bladder carcinoma cells were chosen because they represent a well-established infection model [15] and results therefore are highly reproducible and reliable. We included four *E. coli* isolates from UTI patients of the Freiburg University Hospital and one reference strain (ATCC 11775). Bacteria were inoculated on Columbia 5% sheep blood agar plate (Heipha Dr. Müller GmbH, Germany) and incubated under aerobic conditions at 36 °C for 24 h.

2.3.2. Internalization tests

T-24 cells were grown in 96-well cell culture plates including 100 µl of McCoy 5A medium. Subsequently, 50 µl of bacterial suspension (1 × 10^5 colony forming units [CFU]/ml) and 50 µl of the ITC solution or 50 µl of McCoy 5A medium as control was added and incubated at 37 °C and 5% CO_2. Incubation times were set to 0, 1, 2 and 3 h. All measurements were done eightfold. Subsequently, cell monolayers were washed twice with 200 µl PBS and incubated with 20 µl antibiotic suspension containing 15 µg/ml Gentamicine and 5 µg/ml Imipenem, solved in McCoy's 5 A medium, for 1 h at 36 °C and 5% CO_2 to kill all bacteria not internalized in human cells. After removal of the antibiotics by multiple washing with McCoy 5A medium, 50 µl Trypsin was added to detach the cell monolayer and 50 µl of 2% Triton solution was added. The remaining cell solution was plated onto Mueller Hinton agar plates and incubated for 12 h at 36 °C; CFUs were counted to assess the remaining, thus internalized UPEC.

2.4. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS 20.0, Chicago, USA). Descriptive statistics were used to explore data. The distribution of data was tested with Shapiro-Wilk test. Variables were then analyzed using non-parametric tests. *P*-values of < 0.05 were considered significant. All tests were two-tailed.

3. Results

3.1. Susceptibility testings

The majority of all tested strains 54.5% (121/222) were classified as MDR *E. coli*. The MICs for ITC obtained by broth dilution ranged from < 0.02 to 0.67 mg ITC/ml. The mean overall MIC 90 shows that a concentration of 0.17 mg ITC ml was necessary to inhibit visible bacterial growth in 90% (200 / 217) of all tested strains. Comparing non-MDR to MDR strains, the latter showed significantly higher MIC values (0.16 ± 0.07 mg/ml *versus* 0.18 ± 0.08 mg/ml, $p = .01$) (Fig. 1), although the difference in absolute numbers was rather small.

Fig. 1 shows the relative frequency of different MIC values in MDR and non MDR-strains.

3.2. UPEC internalization

Internalization of UPEC could be reduced by 10 to 67% when treated with ITCs over all incubation periods (Table 1), except for one strain (3279) that actually showed increased internalization when incubated for 1 or 2 h (+30% and + 10.4% respectively).

After 3 h of incubation also this strain showed reduced internalization (13%). Overall, mean reduction of internalization for all strains was highest after 3 h of incubation (41.1%) compared to 1 or 2 h (16.7% and 38%, respectively) of incubation.

4. Discussion

Our data demonstrate a strong antimicrobial activity of ITCs against UPEC represented by very low MIC 90s. Additionally, ITCs are able to inhibit internalization of UPEC into human uroepithelial cells especially after longer periods of incubation. However, this effect is far less consistent than direct antimicrobial activity and appears to differ between strains. Possibly, strains also have varying ability to penetrate human cells since this mechanism depends on many different interactions between surface molecules and results from different genetically determined virulence factors. Unfortunately, we did not evaluate those differing genetic features of our tested strains. However, clinical data