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Synergistic antibacterial effects of β-Chloro-L-alanine and phosphomycin on urinary tract isolates of *E. coli*

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Abstract

Introduction: The aim of this study was the evaluation of the synergistic effect of sub-MIC doses of Phosphomycin with β-Chloro-L-alanine against urinary tract isolates of *E. coli*.

Materials and methods: A total of 40 isolates were collected from urine specimens submitted to the clinical diagnostic Laboratories in Urmia, Iran. The amounts of MIC and MBC for Phosphomycin, β-Chloro-L-alanine or a mixture of 0.5 mM β-Chloro-L-alanine with sub-MIC doses of Phosphomycin were determined and three groups were compared.

Results: Of 40 *E. coli* isolates, 12. 5% were susceptible to all investigated concentrations of phosphomycin and 2. 5% were resistant. The mean MIC value for phosphomycin in the other *E. coli* isolates was determined as 25. 7± 35. 5 µg/ mL. All of the bacterial isolates were resistant to all investigated concentrations of β-Chloro-L-alanine. Application of β-Chloro-L-alanine and phosphomycin combination decreased the MIC and MBC values in 22. 5% of the isolates.

Discussion and conclusion: This study suggests that β-Chloro-L-alanine and phosphomycin combination have *in vitro* synergistic effect on some isolates of urinary tract *E. coli*.

Key words: *E. coli*, β-Chloro-L-alanine, Phosphomycin, Synergistic effect

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Introduction

Urinary tract infections (UTIs) are the most common infectious diseases that occur in the community as well as healthcare setting and *E. coli* is the main agent of this disease (1). Antibiotic therapy is the gold standard for treatment of UTIs, however this microorganism is becoming resistant to the agents that are normally prescribed. This leads to several numbers of management and therapeutic problems. This fact has caused the need to develop new antimicrobial drugs (2- 4).

E. coli is a rod-shaped Gram-negative bacterium and a member of Enterobacteriaceae family. Different types of antibiotics are used for the treatment of UTIs caused by *E. coli* (5). The shortage of new antimicrobial agents has made the scientific community reconsider the potential value of old antibiotics. Phosphomycin is used for single-dose treatment of uncomplicated urinary tract infection due to *E. coli* especially in women (6, 7) but developing bacterial resistance to this antibiotic has limited its usage (8). Many studies showed high efficacy of phosphomycin in treatment of non complicated UTI caused by *E. coli* (9).Guidelines recommended three options for first-line treatment of acute uncomplicated cystitis; including phosphomycin, nitrofurantoin and cotrimoxazole (10), however long term therapies even with very effective antimicrobial drugs may result in numerous side effects and cause selection of resistant bacteria.

Phosphomycin interferes with the bacterial cell wall biosynthesis through the inhibition of UDP-N-Acetyl glucosamine 3 *enolpyruvyl transferase* (MurA) (11). MurA enzyme connects phosphoenol pyruvate (PEP) to the 3-OH group of UDP-N-Acetyl glucosamine that finally leads to the production of one of the peptidoglycan subunits termed Acetyl muramic acid. There are some other chemicals such as phosphinates, thiazolidines and β-Chloro-L-alanine that inhibit peptidoglycan biosynthesis (2, 3, 7, 12 & 13). β-Chloro-L-alanine is a non-toxic amino acid analog that inhibits Alanin-Valine transaminase or transaminase C (MurC) irreversibly. MurC is the enzyme responsible for the assembly of L-alanin to UDP-MurNAC during the intra-cytoplasmic stage of peptidoglycan biosynthesis (13). It seems that two peptidoglycan biosynthesis inhibiting agents have synergetic effect as they used in combination. The synergistic activities of phosphomycin with β -lactam antibiotics as well as β-Chloro-L-alanine with methicillin have been reported previously (14). Phosphomycin and β-Chloro-L-alanine both inhibit the intra-cytoplasmic stages of peptidoglycan biosynthesis and in respect to rapid occurrence of resistance to phosphomycin, its clinical usage becomes limited (15), in order to overcome to this disadvantage of phosphomycin, we tested the hypothesis that sub-MIC doses of Phosphomycin as a Mur A inhibitor antibiotic, may have synergistic effects in combination with β-Chloro-L-alanine as a safe Mur C inhibitor on UTIs isolates of *E. coli*.

Material and Method

Bacterial isolates and culture media

A total of 40 isolates were collected from urine specimens submitted to the clinical diagnostic laboratories in Urmia, Iran during a 12 months period from March 2010 to February 2011. The isolates were further processed by standard methods (16) to identify as *E. coli*. Isolated bacteria was maintained for long storage on skim milk medium (BBL; Becton Dickinson Microbiology Systems Cockeysville, MD21030, U. S. A). by adding 10% glycerol in -80°C. *E. coli* (ATCC25922) was used as the reference strain (17).

Determination of antibacterial activity of phosphomycin and β-Chloro-L-alanine

The frozen bacterial isolates were inoculated on nutrient agar medium and then cultured overnight at 36±0. 5°C. The bacteria were suspended in sterile buffer saline and used as inoculate within one hour after adjustment. Bacterial inoculate were added to serial dilutions of phosphomycin (Sigma-Aldrich) or β-Chloro-L-alanine (Sigma-Aldrich), with final bacterial concentration of 1. 5×10^6 cell /mL by adjusting with *Mc Farland's Turbidity Standard* No. 0. 5. MICs and MBCs of phosphomycin and β-Chloro-L-alanine against tested isolates were determined by dilution method using Mueller Hinton Broth (BBL) according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2006). The range concentration for determining MIC and MBC values for phosphomycin and β-Chloro-L-alanine was considered as 0. 25-128 mg/Land 0. 0625- 1 mM respectively. The experiments were carried out in triplicate. The MIC and MBC values were defined as the lowest antibiotic concentration that completely prevented turbidity in broth or colony growth on agar medium after incubation at 37°C for 24 hour respectively (18).

Determination of synergetic activity of phosphomycin with β-Chloro-L-alanine

Nutrient broth medium with sub-MIC concentrations of phosphomycin for each isolate was prepared, fixed concentrations of β-Chloro-L-alanine (0. 5 mM) was added to each tube. 1. 5×10^6 CFU/mL of each isolate were inoculated in each tube and incubated at 36±0. 5°C. After overnight incubation, MIC and MBC of phosphomycin in the presence of 0. 5 mM β-Chloro-L-alanine were determined (19). Synergy was described as if there was a reduction in MIC (or MBC) of phosphomycin in the presence of 0. 5 mM of β-Chloro-L-alanine for each isolate (20).

Results

From 40 *E. coli* isolates, only 12. 5% were susceptible to all investigated concentrations of phosphomycin and 2. 5% were resistant, so in the selected range of phosphomycin concentrations, we could determine MIC and MBC values for 85% of isolates. The mean MIC value for phosphomycin in the other isolates was determined as 25. 7± 35. 5 mg /L, all the bacterial isolates were resistant to investigated concentrations of β-Chloro-L-alanine. In vitro application of β-Chloro-L-alanine and phosphomycin combination decreased the MIC as well as MBC values in 22. 5% of the isolates (Fig. 1 and 2).

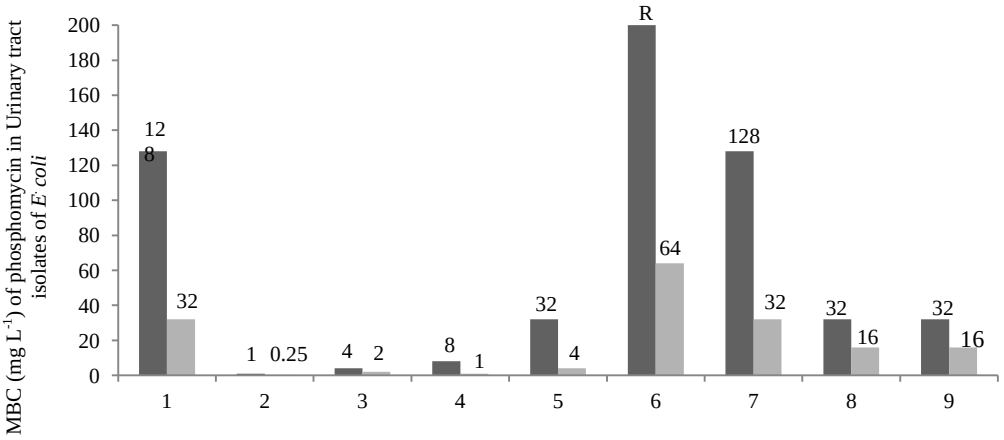

Fig. 1- Reduction of phosphomycin MBC in nine *E. coli* isolates.

As demonstrated in Fig. 1, β-Chloro-L-alanine has significantly decreased the MBC values of phosphomycin for nine isolates. (Black: MBC of phosphomycin for each isolate, Gray: MBC of phosphomycin in the presence of 0. 5 mM β-Chloro-L-alanine for each isolate).

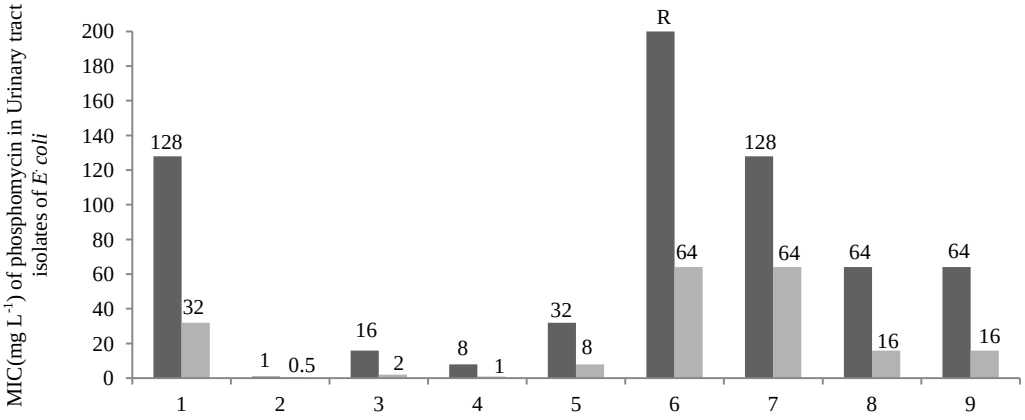

Fig. 2- Reduction of phosphomycin MIC in nine *E. coli* isolates.

As demonstrated in Fig. 2, β-Chloro-L-alanine has significantly decreased the MIC values of phosphomycin for nine isolates. (Black: MIC of phosphomycin for each isolate, Gray: MIC of phosphomycin in the presence of 0. 5 mM β-Chloro-L-alanine for each isolate).

Discussion and Conclusion

Phosphomycin has been used in the treatment of lower urinary tract infections and also systemic infections caused by Gram-negative bacteria in the recent years. Marchese *et al.* showed that phosphomycin is the most effective antibiotic in the treatment of non-severe urinary tract infections caused by *E. coli* and 99% of *E. coli* isolates were sensitive to this antibiotic (21). Also Fabre *et al.*. showed that 98% of the *E. coli* isolates were sensitive to phosphomycin and concluded that this drug is one of the most effective antibiotics in the treatment of non-nosocomial urinary infections (22). Heising et al. showed that the resistance of UTI agents to phosphomycin is less than 6% in Europe, indicating that this drug can be a good choice for the blind treatment of UTIs, however the administration of phosphomycin during the medication courses can cause a rapid development of resistance to it (21, 23). Phosphomycin has attracted renewed interest for the treatment of UTIs caused by gram-negative bacilli with resistance to traditionally used antibiotics. The main concern regarding the clinical utility of phosphomycin refers to the potentiality for the emergence of resistance during therapy. Mutants that are resistant to phosphomycin are developed rapidly in vitro. Several studies have assessed the frequency of mutation to phosphomycin resistance for gram-negative bacteria (24).

In many cases, using a combination of two or more antibiotics reduces the risk of developing antibiotic resistant strains. So in this study we tried to examine antibacterial effects of a combination of phosphomycin and β-Chloro-L-alanine on *E. coli* isolates in order to introduce an effective combination drug including phosphomycin for treatment of uncomplicated UTIs. Zoeiby *et al.*showed that β-Chloro-L-

alanine is the inhibitor of MurC enzyme of *E. coli* in a way that they interfere with the L-alanine (forth amino acid in tetra-peptide structure of bacterial peptidoglycan) during peptidoglycan biosynthesis (2).

Martinez et al. showed that phosphomycin has no in vitro effects on 34 clinical isolates of *Pseudomonas auroginosa*, however it showed synergistic effects in combination with tobramycin or amikacin on the same isolates (25). It has been already shown that D-cycloserine is one of the inhibitors of peptidoglycan biosynthesis, although this antibiotic is so effective in the treatment of tuberculosis, its high toxicity has limited its usage. Administration of β-Chloro-L-alanine along with D-cycloserine decreased the MIC of cycloserine against *Mycobacterium tuberculosis*. This data shows the administration of these agents together may decrease the dosage of both drugs and may be significantly effective in bacterial growth inhibition (26). Browski *et al*. showed the synergistic activity of phosphomycin with ampicillin and streptomycin on 70% of *E. coli* isolates. The authors postulate that a combination of phosphomycin with beta-lactam or aminoglycoside antibiotics may be used in clinical practice and such a procedure should prevent an emergence of phosphomycin-resistant strains (27).

The results of this study showed the relative efficiency of β-Chloro-L-alanine in decreasing MIC and MBC values of phosphomycin against 22. 5% of urinary tract *E. coli* isolates. This is the first report to our knowledge that shows the relative efficiency of β-Chloro-L-alanine in decreasing MIC and MBC values of phosphomycin against some urinary tract *E. coli* isolates. Therefore we suggest that the combination of β-Chloro-L-alanine and phosphomycin may be at least partially effective in treating of UTIs, however more studies on numerous urinary tract isolates of *E. coli* as well as *in vivo* studies not only in experimental animals but also in human should be done for confirmation of the synergistic antibacterial effect of β-Chloro-L-alanine in combination with phosphomycin for treatment of UTIs caused by *E. coli*.

References

(1) Pallett A, Hand K. Complicated urinary tract infections: practical solutions for the treatment of multi resistant Gram-negative bacteria. *J Antimicrob Chemother*. 2010; 65 Suppl3: iii25-33.

(2) El Zoeiby A, Sanschagrin F. Structure and function of the mur enzymes: development of novel inhibitors. *Mol Microbiol*. 2003; 47(1): 1-12.

(3) Silver LL. Novel inhibitors of bacterial cell wall synthesis. *Curr Opin Microbiol*. 2003; 6(5): 431-38.

(4) Silver LL. Does the cell wall of bacteria remain a viable source of targets for novel antibiotics? *Biochem Pharmacol*. 2006; 71(7): 996 - 1005.

(5) Brooks G, Carroll KC, Butel J, Morse S. Jawetz *Melnick and Adelbergs Medical Microbiology*. 25nd ed, USA, Mc-Graw Hill, 2010; 249- 62.

(6) Allerberger F, Klare I. In-vitro activity of phosphomycin against vancomycin-resistant enterococci. *J Antimicrob Chemother*. 1999; 43: 211– 17.

(7) Falagas ME, Roussos N, Gkegkes ID, Rafailidis PI, Karageorgopoulos DE. Phosphomycin for the treatment of infections caused by Gram-positive cocci with advanced antimicrobial drug resistance: a review of microbiological, animal and clinical studies. 2009; *Expert Opin Invest Drugs*. 2009; 18: 921- 44.

(8) de Jong Z, Pontonnier F, Plante P. Single-dose phosphomycin trometamol (Monuril) versus multiple-dose norfloxacin: results of a multicenter study in females with uncomplicated lower urinary tract infections. *Urol. Int*. 1991; 46:344- 48.

(9) Ching-Lan L, Chia-Ying L, Yu-Tsung HuangChun-Hsing L, Lee-Jene T, Turnidge JD, Hsueh PR. Antimicrobial Susceptibilities of Commonly Encountered Bacterial Isolates to phosphomycin determined by Agar Dilution and

Disk Diffusion Methods. *Antimicrob Agents Chemother*. 2011; 55(9): 4295- 301.

(10) Colgan R, Williams M. Diagnosis and treatment of acute uncomplicated cystitis. *Am Fam Physician*. 2011; 84(7):771- 76.

(11) Whalen WA, Wang MD, Berg CM. beta-Chloro-L-alanine inhibition of the *Escherichia coli* alanine-valine transaminase. *J Bacteriol.* 1985; 164(3):1350- 52.

(12) Grif K, Dierich MP, Pfaller K, Miglioli PA, Allerberger F. In vitro activity of phosphomycin in combination with various anti-staphylococcal substances. *J Antimicrob Chemother*. 2001; 48(2): 209- 17.

(13) Mobashery S, Johnston M. Inactivation of alanine racemase by beta-chloro-L-alanine released enzymatically from amino acid and peptide C10-esters of deacetyl cephalothin. *Biochem*. 1987; 26(18): 5878- 88.

(14) Hoşbul T, Ozyurt M, Baylan O, Bektöre B, Ardiç N, Ceylan S, Erdemoğlu A, Haznedaroğlu T. In vitro activity of phosphomycin trometamol in the treatment of *Escherichia coli* related uncomplicated urinary tract infections. *Mikrobiyol Bul*. 2009; 43(4):645- 49. Article in Turkish.

(15) Smith CA. Structure, function and dynamics in the mur family of bacterial cell wall ligases. *J Mol Biol*. 2006; 362(4): 640- 55.

(16) Forbes BA, Sahm DF, Weissfeld AS. Bailey & Scott's Diagnostic Microbiology. 12nd ed, St. Louis, Missouri: Mosby, 2007.

(17) Jazani NH, Shahabi Sh, Ali AA. Antibacterial effects of water soluble green tea extracts on multi-antibiotic resistant isolates of *Pseudomonas aeruginosa*. *Pak J Biol Sci*. 2007; 10(9):1544- 46.

(18) Jazani NH, Zartoshti M, Shahabi Sh, Yekta Z, Nateghi S. Evaluation of the synergetic effects of water soluble extracts of green tea (Camellia sinensis) on the activity of ciprofloxacin in urinary isolated *E. coli* . *J Biol Sci*. 2007; 7(8): 1500- 503.

(19) Tiwari TP, Bharti SK, Kaur HD, Dikshit RP, Hoondal GS. Synergistic antimicrobial activity of tea and antibiotics. *Ind JMed Res*. 2005;122: 80- 84.

(20) Aboulmagd E, Hamdan I. Al-Badry A, Al-Badry S. Synergism and post antibiotic effect of green tea extract and imipenem against methicillin-resistant *Staphylococcus aureus*. *Microbiol Jour*. 2011; 1: 89- 96.

(21) Marchese A, Gualco L, Debbia EA, Schito GC, Schito AM. In vitro activity of phosphomycin against gram-negative urinary pathogens and the biological cost of fosfomycin resistance. *Mikrobiyol Bul.* 2009; 43(4): 645- 49.

(22) Fabre R, Mérens A, Lefebvre F, Epifanoff G, Cerutti F, Pupin H, et al. Susceptibility to antibiotics of *Escherichia coli* isolated from community-acquired urinary tract infections *Med Mal Infect.* 2010; 40(10): 555- 59.

(23) Heisig P. Urinary tract infections and antibiotic resistance. *Urologe A*. 2010; 49(5): 612- 17.

(24) Karageorgopoulos D, Wang, Xu-hong Yu R,Falagas M. Phosphomycin: evaluation of the published evidence on the emergence of antimicrobial resistance in Gram-negative pathogens. *J Antimicrob Chemother* 2012; 67(2): 255- 68.

(25) Martinez-Martinez L, Rodriguez G, Pascual A, Suarez AI, Perea EJ. In vitro activity of antimicrobial agent combinations against multi-resistant *Acinetobacter baumannii* . *J Antimicrob Chemother* 1996; 38(6): 1107- 8.

(26) David S. Synergic activity of D-cycloserine and beta-chloro-D-alanine against *Mycobacterium tuberculosis*. *J Antimicrob Chemother*. 2001; 47(2): 203- 6.

(27) Borowski, J, Linda H. Combined action of fosfomycin with beta-lactam and aminoglycoside antibiotics. *Chemother*. 1977; 23 Suppl 1: 82- 5.

اثرات سینرژیتیک ضد باکتریایی بتاکلروال-آلانین و فسفومایسین بر روی جدایه‌های ادراری اشرشیاکلی

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چکیده

مقدمه: هدف مطالعه حاضر، بررسی اثر سینرژیتیک دوزهای Sub-MIC فسفومایسین همراه با بتا کلروآلانین بر روی جدایه‌های ادراری اشرشیا کلی است.

مواد و روش‌ها: تعداد ۴۰ جدایه از نمونه های ادراری ارسالی به آزمایشگاه‌های تشخیص طبی ارومیه، آذربایجان غربی جداسازی شدند. مقادیر MIC و MBC درمورد فسفومایسین، بتاکلروال آلانین و مخلوطی از ۰/۵ میلی‌مولار بتا کلروال- آلانین و دوز های Sub-MIC فسفومایسین تعیین شد و سه گروه مقایسه شدند.

نتایج: از ۴۰ جدایه اشرشیاکلی، ۱۲/۵ درصد نسبت به تمامی غلظت‌های مورد مطالعه از فسفومایسین حساس و ۲/۵ درصد مقاوم بودند. میانگین MIC برای فسفومایسین درمورد سایر جدایه های اشرشیاکلی ۳۵/۵ ± ۲۵/۷ میکروگرم در سی‌سی تعیین شد. کاربرد مخلوط بتا کلروال آلانین و فسفومایسین مقادیر MIC وMBC را در مورد ۲۲/۵ درصد از جدایه‌ها کاهش داد.

بحث و نتیجه‌گیری: نتایج این مطالعه پیشنهاد می‌کند که مخلوط فسفومایسین و بتا کلرو ال آلانین دارای اثرات سینرژیتیک بر روی برخی ازجدایه‌های ادراری اشرشیاکلی است.

واژه‌های کلیدی: اشرشیاکلی، بتاکلروال-آلانین، فسفومایسین، اثرات سینرژیتیک

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