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Influence of cyclosporine A and quercetine on MIC values of danofloxacin mesylate in *Escherichia coli* strains isolated from poultry

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Abstract: A reference strain *Escherichia coli* ATCC 25922, sensitive and resistant strains of *E. coli*, isolated from chickens with colibacillosis were tested. The aim of the present study was to determine the effect of modulators of ABC efflux transporters in eukaryotic cells cyclosporine A and quercetine on MIC values of danofloxacin mesylate. MIC values of danofloxacine mesylate against the chosen strains were determined with or without modulators at concentrations of 25 µM, 5 µM, 1 µM, and 0.2 µM by macro broth dilution method. Results show that in the presence of cyclosporine A, MIC values decreased from 2-fold to 16-fold in the investigated strains of *E. coli.* Combination of danofloxacin mesylate and quercetine resulted in an increase of MIC values. The observed changes in the values of MICs suggest participation of ATP-dependent efflux of danofloxacin mesylate from bacterial cells.

Key Words: Danofloxacin mesylate, cyclosporine A, quercetine, *Escherichia coli*

Escherichia coli is a major pathogen of worldwide importance in commercially produced poultry contributing significantly to economic losses. It causes colibacillosis and airsacculitis in poultry which can result in high morbidity and mortality. Danofloxacin mesylate is a synthetic antibacterial agent of the fluoroquinolone class used for treatment of bacterial diseases of cattle, swine, and poultry, including *E. coli* infections. Fluoroquinolones are among the most recently approved classes of antimicrobial agents but resistance has already been documented (1).

Bacteria develop resistance to fluoroquinolones through at least 2 different mechanisms. Mutations in *gyrA* and *parC* genes that encode target DNA gyrase and topoisomerase IV are the best-known resistance mechanism (2) but mutations that lead to expression of efflux pumps to enhance excretion of quinolones out of the bacterial cell are becoming increasingly common (3).

ATP-dependent efflux pumps were found in bacteria to mammalian species. Most of the eukaryotic multidrug efflux pumps belong to the ATP

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binding cassette (ABC) family of transport proteins, while in the prokaryotic systems they are mainly secondary transporters. Some efflux pumps selectively extrude specific antibiotics, while others, referred to as multidrug resistance (MDR) pumps, expel various structurally diverse antibiotics (4). Three transporters, AcrAB, MdfA, and YdhE, have been shown to efflux quinolones in *E. coli*. The AcrAB pump is a member of the RND family (resistance-nodulation-division), MdfA is a member of the MFS (major facilitator superfamily) and YdhE is the founding member of the most recently identified MATE (multidrug and toxic compound extrusion) family of pumps (5). Two ABC multidrug transporters, LmrA of *Lactococcus lactis* and HorA of *Lactobacillus brevis,* in gram-positive bacteria have been characterized thus far (6). Kobayashi et al. (7) reported a macrolide specific ABC transporter in *E. coli*. VcaM of Non-O1 *Vibrio cholerae* represents the first ABC multidrug efflux pump in gram-negative bacteria (8).

In the last decade, the modulators of the function of ABC transporters in eukaryotic cells were widely studied. Cyclosporine A is not a specific and very potent inhibitor of ABC transporter proteins. Quercetine, a flavonoide, is recognized as inhibitor or inductor of these efflux pumps, depending on the used concentration (9,10).

It was suggested that combination of antibacterial drugs with inhibitors of ABC transporters could be useful in treatment of bacterial infections. Data about the effect of the modulators of ABC transporters of eukaryotic cells on the efflux proteins in prokaryotic cells are still very limited. Therefore, the aim of the current study was to investigate the effect of cyclosporine A and quercetine on the MIC values of danofloxacin mesylate for *E. coli* strains isolated from poultry.

Bacterial strains: Following strains were included in the current study: a reference strain *E. coli* ATCC 25922 and SEPEC *E. coli* (resistant-R and sensitive-S strains) isolated from tissues of chickens at different ages. The material from the investigated tissues (heart, liver, lung, and spleen), obtained from fresh carcasses were cultivated on Tryptic Soy Agar (Becton Diskinson) with addition of 5% defibrinated sheep blood and on McConkey agar. They were incubated aerobically at 37 °C for 24-48 h. Colonies, which show lactose-positive and oxidase-negative results, were identified by BBL Crystal™ Identification Systems, Enteric/Nonfermentor ID Kit (Becton Diskinson). *E. coli* strains were serotyped by using group- and type-specific sera (manufactured from NCIPD, Sofia, Bulgaria) by slide agglutination techniques.

Chemicals (modulators) and drugs: Danofloxacin mesylate was obtained from Pfizer (Sandwich, UK). Cyclosporine A and quercetine dehydrate minimum 98% HPLC grade were obtained from Sigma.

Determination of minimum inhibitory concentrations (MIC) of danofloxacin mesylate against E. coli strains: Minimum inhibitory concentration (MIC) of danofloxacin mesylate against *E. coli* strains was determined by Broth Macrodilution Method (11). Cation-adjusted Mueller-Hinton broth (Becton Diskinson) was used for susceptibility tests. The tested strains were classified as resistant (R) if their MIC was higher or identical with breakpoint of 2 μg/mL; as intermediate – with MIC 0.5-1 μg/mL and as sensitive – with MIC ≤0.25 μg/mL.

Trials with modulators of ABC efflux transporter proteins: Modulators for ABC efflux transporters cyclosporine A and quercetine were applied at the following concentrations: 25 μM, 5 μM, 1 μM, and 0.2 μM. Influence of these compounds on *E. coli* strains was established by determination of MIC values of danofloxacin mesylate in combination with cyclosporine A or quercetine. The investigated strains were chosen according to their MIC values: sensitive (O78:K80) with MIC of 0.25 μg/ml and resistant with MIC of 32 μg/ml. MIC values of danofloxacin mesylate against the tested strains (without and with modulators) were determined 4-fold. As a reference control was used *E. coli* ATCC 25922. MIC value of danofloxacin mesylate with/without modulators was determined as the lowest concentration, at which growth of bacteria was not observed.

The values of MIC of danofloxacin mesylate against the tested strains *E. coli*, without or with cyclosporine A at different concentrations are shown in Table 1. MIC values were decreased in combination with this compound. Cyclosporine A at 5 μM decreased to the lowest degree (16-fold) MIC value against the reference strain. At 1 μM and 0.2 μM, this

Table 1. MIC values of danofloxacin mesylate (DM) with or without cyclosporine A at different concentrations against *E. coli* ATCC 25922, sensitive (S) and resistant (R) strains of *E. coli*.

E. coli	DM	DM + cyclosporine A			
	MIC μg/ml	25 μM	5 μM	1 μM	0.2 μM
E. coli ATCC 25922	0.03	0.015	0.002	0.008	0.008
E. coli /S/ O78:K80	0.25	0.125	0.125	0.125	0.125
E. coli / R /	32	16	32	16	32

agent lowered MIC value 4-fold for *E. coli* ATCC 25922. All of the tested concentration, Cyclosporine A decreased MIC value against the sensitive *E. coli* O78:K80 strain 2-fold but MIC values of the resistant strain were influenced to a lesser degree.

The effect of quercetine on MIC values of danofloxacin mesylate was controversial (Table 2). Four-fold decreasing of MIC value against the reference strain *E. coli* ATCC 25922 was determined when quercetine was applied at the concentration of 1 μM. Low or high concentrations of quercetine used did not change MIC values against the resistant strain. They even increased the values of MIC against the sensitive and reference strains 2-fold. *E. coli* genome encodes 57 ABC-transporters that occupy almost 5% of the genome (12) but their role in drug transport is not well understood, yet. It was demonstrated that fluoroquinolones, including danofloxacin mesylate, are among substrates for ABC transporter proteins in eukaryotic cells (13). Significance of the ATP-dependent efflux of fluoroquinolones in sensitive strain *Escherichia coli KAM32* was proved by Lee et al. (14). Expression of EfrAB (transport efflux protein) in this strain contributes to 4-fold increasing of MIC values of ciprofloxacin and norfloxacin. Involvement of these proteins in drug transport at a functional level is demonstrated by studies with broad and specific inhibitors. Verapamil, a potent inhibitor for ABC efflux transporter proteins of eukaryotic cells, decreases acriflavine efflux by 50% in *Escherichia coli KAM32* with induced expression of EfrAB. Transformation of the hypersensitive *E. coli* strain CS1562 by a LmrA encoding plasmid resulted in an increased resistance to 17 out of 21 clinically most used antibiotics, including fluoroquinolones (15). Cyclosporine A showed inhibitory activity on ATP-dependent efflux proteins in eukaryotic cells. Ex vivo experiments with chicken lymphocytes showed that cyclosporine A at a concentration of 4 μM significantly inhibits efflux of fluorescent dye Rhodamine 123 (Rh-123), which is a good substrate for P-glycoprotein, applied at a concentration of 0.5

Table 2. MIC values of danofloxacin mesylate (DM) with or without quercetine at different concentrations against *E. coli* ATCC 25922, sensitive (S) and resistant (R) strains of *E. coli*.

E. coli	DM	DM + cyclosporine A			
	MIC μg/ml	25 μM	5 μM	1 μM	0.2 μM
E. coli ATCC 25922	0.03	0.06	0.06	0.008	0.03
E. coli /S/ O78:K80	0.25	0.5	0.25	0.25	0.5
E. coli / R /	32	32	32	32	32

μM (16). Very high concentration of cyclosporine A (20 μM) did not change Rh-123 efflux significantly. In contrast to chickens, 20 μM cyclosporine A significantly inhibited Rh-123 efflux in mammalian lymphocytes. Cyclosporine A inhibits Rh-123 efflux at the highest degree in goats, human being, cattle and sheep, and to a lower degree in pigs and horses. Lack of inhibitory activity was determined in rats (9). Our data from these functional studies suggest that this compound exert also inhibitory effects in prokaryotic cells. Published data and our results suggest participation of ATP-dependent efflux of danofloxacin mesylate in the investigated strains *E. coli*.

Flavonoides, including quercetine, inhibit or induce ABC efflux transporter proteins in eukaryotic cells in a concentration dependent manner. They could restore the sensitivity of cancer cells to anticancer drugs by inhibition of function of P-glycoprotein, MRP1 (multidrug-resistance protein 1), and BCRP (breast cancer resistance protein). Quercetine stimulate the efflux of substrates of P-glycoprotein at low concentrations (5-10 μM) but it inhibits this transport at high concentrations (50 μM) (17). It could modulate the efflux P-glycoprotein also in dependence of the substrates. For example, it inhibits the efflux of Rh-123 and increases the efflux of doxorubicin (18). It was also demonstrated that quercetine itself could inhibit supercoiling activity of bacterial gyrase and induces DNA cleavage in *E. coli* (19). Therefore, we studied different concentrations of quercetine on MIC values of danofloxacin mesylate against strains of *E. coli*. Our results suggest that there is no significant interaction between danofloxacin mesylate and quercetine at a functional level in the investigated strains of *E. coli*. A tendency of increase in the values of MIC of danofloxacin mesylate by quercetine could be explained by the potentiated efflux of the antimicrobial agent. Opposite effect of flavonoids was observed in gram-positive microorganisms. These compounds potentiate activity of ciprofloxacin and norfloxacin against *Staphylococcus aureus* by inhibition of bacterial multidrug resistance pumps (20). These results allow us to conclude that the effect of flavonoides in gram-negative microorganisms could differ from those in gram-positive bacteria.

In conclusion, as a first investigation of influence of modulators of ABC transport proteins, cyclosporine A and quercetine in *E. coli* from poultry origin, it requires to be extended. These functional trials could be added with studies of the level of expression of these proteins and multidrug resistance proteins from other families as SMR, MATE, RND, and MFS. Further investigations and discovery of proper inhibitors of these multidrug efflux pumps, applied in combination with antimicrobial drugs, could improve the outcome of antibacterial therapy. It also could decrease the risk of selection of resistant subpopulation microorganisms. Determination of the efflux proteins from ABC family and at transcriptional level could give additional information about their role in the selection of resistance against antibacterial drugs.

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