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Impact of cyadox on human colonic microflora in chemostat models

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ABSTRACT

The aim of this study was to evaluate the microbiological safety of cyadox, a new member of quinoxaline-1,4-dioxides (QdNOs), on human intestinal flora. Four chemostats containing human fecal flora were exposed to 0, 16, 32, and 128 μg/mL of cyadox, respectively. Bacterial populations, resistance rates of two predominant bacteria and short-chain fatty acids (SCFA) were monitored daily prior to and during drug MOA Laboratory of Risk Assessment for Quality and Safety of Livestock and Poultry Products exposure. Colonization resistance (CR) of each community was determined by three successive daily challenges of *Salmonella typhimurium*. Efflux pump gene (*oqxAB*) in the *Escherichia coli* and *Enterococcus* strains were analyzed by PCR amplification and DNA sequencing. No change in SCFA was observed after exposure to different concentrations of cyadox. Lower concentration of cyadox (16 μg/mL) had no adverse effect on human microflora. However, higher concentrations of cyadox (32 and 128 μg/mL) could change bacterial population and increase the proportion of resistant *E. coli* and *Enterococcus*. More than 26% (12/46) of cyadox resistant *E. coli* strains contained *oqxAB* gene, while all the resistant *Enterococcus* were negative to *oqxAB* gene. Relationship between the occurrence of *oqxAB* gene and cyadox exposure is inconclusive. Our data indicated that 16 μg/mL might be the no observed effect concentration (NOEC) of cyadox. Derived microbiological acceptable daily intake (mADI) would be 1552.03 μg/kg d. The data obtained in present study indicated that cyadox was a safe member of QdNOs family of antimicrobial agents.

1. Introduction

Cyadox is a new member of quinoxaline-1,4-di-N-oxides (QdNOs) family of antibacterial agents. Pharmacological data showed that cyadox had a broad range of antimicrobial spectra and could improve animal production performance (Dvorak, 1984). Toxicological data indicated that cyadox had much lower level of toxicity and higher safety when compared with early products of QdNOs including carbadox and olaquindox (Cihak and Srb, 1983; Fang et al., 2006; He et al., 2006; Nunoshiba and Nishioka, 1989; Wang et al., 2011a,b). Following with the metabolism and residue depletion study, several metabolites (cy1, cy2, cy4, cy6, cy10 and cy12) were identified from animal body in a recent research (Xu et al., 2011). As a new member of QdNOs, cyadox may have a better developing potential and have a wider foreground. However, the microbiological safety of cyadox and its metabolites has never been evaluated.

According to the recommendation of Food and Drug Administration (FDA), microbiological safety evaluation is an essential part of safety assessment for a veterinary drug (FDA, 2005). For establishing a microbilogical acceptable daily intake (mADI), following microbiological endpoints related to current public health concern should be considered: (1) disruption of colonization barrier, (2) increase in the population of resistant bacteria (FDA, 2005). In intestinal tract, four predominant bacteria (*Escherichia coli*, *Enterococcus*, *Bifidobacterium*, *Bacteroides fragilis*) of the normal intestinal floras may play an important role in developing colonization barrier against exogenous microorganisms (e.g. *Salmonella typhimurium*). Population of the predominant flora and the colonization of exogenous *S. typhimurium* were the indices for evaluating the effect of antimicrobial agents on colonization resistance (FDA, 2005). The microbial communities of intestine could produce SCFA which were believed to play an important role in stabilizing the intestinal environment and maintaining colonic health (Kaji et al., 2011; Tobe et al., 2011). Among the *in vitro* and *in vivo* models recommended by FDA, chemostat model was a good option as *in vitro* continuous culturing model which could closely imitate human intestinal microbial environment (FDA, 2005). The chemostat model has been used for evaluating the impact of drug residues on

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human colonic microflora in several studies (Carman et al., 2005; Carman and Woodburn, 2001).

Since the increase in resistance rate of the intestinal bacteria is an important microbiological endpoint of current public health concern, it is necessary to investigate the development and mechanism of cyadox resistance in the intestinal bacteria. Previous studies indicated that *oqxAB* genes might mediate the QdNOs' resistance (Guo et al., 2012). The *oqxAB* genes located in *pOLA52* plasmid were firstly found in the resistant *E. coli* isolated from swine after a long term usage of olaquindox as an antibacterial growth promoter (Sorensen et al., 2003). A recent epidemiological study found that 39% of *E. coli* isolates carried *oqxAB* genes (Zhao et al., 2010). It is unknown whether the cyadox can affect the development of resistance and the emergence of *oqxAB* genes or not.

Although systematic toxicological research has been carried out to evaluate the efficiency and safety of cyadox in our lab, the microbiological safety of this drug has never been assessed. It is also unknown whether cyadox has adverse effect on human colonic microflora or not. The no observed effect concentration (NOEC) and the microbiological acceptable daily intake (mADI) have never been calculated based on microbiological safety of cyadox. In an effort to provide a complete safety spectrum of cyadox, this study was performed to investigate its effect of cyadox on human colonic microflora using a chemostat model and to establish a microbiological NOEC and mADI values for the new veterinary drug (cyadox) in human food.

2. Materials and methods

2.1. Chemicals

Cyadox and its metabolites (cy1, cy2, cy4, cy6, cy10 and cy12) were already synthesized in our lab and dissolved in dimethyl sulfoxide (DMSO). The quinocetone (QUI) was dissolved by DMSO and then diluted with deionized water. The olaquindox (OLA), mequindox (MAQ), cefotaxime (CTX), enrofloxacin (ENR), tetracycline (TET) and chlortetracycline (CHL) were dissolved in deionized water, while amoxicillin (AML) was dissolved in phosphate buffer solution (PBS). All of the chemicals were prepared at a concentration of 1280 μg/mL and kept as stock solution at −20 °C.

2.2. Bacterial srtains

E. coli ATCC 25922, *E. faecalis* ATCC29212 and *B. fragilis* 25285, purchased from American Type Culture Collection (ATCC), were used as quality controls for minimum inhibitory concentration (MIC) determination and species identification. The OqxAB positive *E. coli* was kindly donated by South China Agricultural University and used as a positive control in PCR amplification of o*qxAB* gene. Ciprofloxacin resistant *S. typhimurium* SI3 was obtained from previous study (Sun et al., 2011) and used as a challenge material for the evaluation of colonization resistance.

2.3. Selection of cyadox concentration in preliminary tests

Four adult volunteers (two males and two females, 18–40 years old) were selected with no history of gastrointestinal disturbance or antibiotic usage within the preceding 3 months. Fresh fecal samples from the volunteers were passed directly into anaerobic bags (BioMérieux) and stored at 4 °C for up to 60 min. These samples were then pooled and uniformly supplemented with pre-reduced anaerobically sterilized (PRAS) phosphate buffered saline (PBS buffer) for the preparation of 20% (w:v) suspensions. Prepared fecal samples were then used in preliminary tests and in chemostat models.

From the prepared fecal samples, four predominant intestinal bacteria (*E. coli*, *Enterococcus*, *Bifidobacterium* and *B. fragilis*) were isolated by selective agars according to the microbiology laboratory guidebooks published by ministry of health in China (2003). Eosin methylene blue agar medium (EMB), bile esculin azide agar (BEA), BBL medium and bacteroides bile esculin agar (BBE) were used for the selective culture of *E. coli*, *Enterococcus*, *bifidobacerium* and *B. fragilis*, respectively. Species identification was confirmed by classic biochemical tests and ABI 3130 system (Applied Biosystem, USA).

Representative strains isolated from fresh fecal samples were subjected to minimum inhibitory concentration (MIC) determination under anaerobic condition (85%N_2, 10%H_2 and 5%CO_2) according to the methods defined in CLSI (CLSI, 2009, 2010). MICs of cyadox and its metabolites against *E. coli* and *Enterococci* were determined by microbroth dilution test according to the protocol given in CLSI document M100-S19E (CLSI, 2010). MICs of cyadox and its metabolites against *Bifidobacterium* and *B. fragilis* were determined by the agar dilution method described in CLSI document M11-A4 (CLSI, 2009).

A series of 2 fold increased concentrations of cyadox (0, 0.5, 1, 2, . . .,128 μg/ml) were mixed with the prepared fecal samples and then incubated in an anaerobic glove box at 37 °C for 24 h. Populations of the four different bacteria (*E. coli*, *Enterococcus*, *Bifidobacterium* and *B. fragilis*) were counted by selective agars. Resistant *E. coli* and *Enterococci* were enumerated respectively by EMB and BEA selective agars containing 4-fold MIC of cyadox. Resistance rate of *E. coli* and *Enterococci* was the number of resistant colonies divided by the total number of each strain.

2.4. Establishment of chemostat model

Culture medium was prepared according to previously published papers with slight modifications (Carman et al., 2005; Carman and Woodburn, 2001). Modified culture medium contained starch (5.0 μg/ml), bovine milk casein (3.0 μg/ml), peptone (3.0 μg/ml), amylopectin (0.6 μg/ml), pectin (0.6 μg/ml), xylan (0.6 μg/ml), L-cysteine (0.4 μg/ml), cholesterol (0.25 μg/ml), chenodeoxycholic acid (0.25 μg/ml), cholic acid (0.25 μg/ml), hemin (0.01 μg/ml), KH_2PO_4 (2.0 μg/ml), $NaHCO_3$ (0.1 μg/ml), NaCl (4.5 μg/ml), MgSO4·$7H_2O$ (0.5 μg/ml), and $CaCl_2$·$2H_2O$ (0.45 μg/ml).

Four separate chemostats were run in parallel for simulating human colonic ecosystem. A 500 mL culture vessel was maintained under fixed conditions (37 °C, nitrogen atmosphere and pH 6.4–6.6). Prepared fecal suspension of 50 mL (See 2.3) was inoculated into each culture vessel by injecting through a septum using syringes. After 7 h incubation, culture medium was pumped into and out of the culture vessel at a uniform rate of 35 mL/h to maintain 500 mL of final culture medium. After 7 days' running, the chemostats reached to a steady state.

2.5. Assessment of microbiological safety of cyadox using chemostat models

From 7th to 13th day, the chemostats were kept on running without administration of cyadox. From 14th to 20th day, three concentrations of cyadox, selected from preliminary test, were infused into the culture medium. Before and during the administration of cyadox (7–20th day) into the chemostat model, 2 mL of the samples was taken out from the chemostat models every day. Population of the four predominant bacteria (*E. coli*, *Enterococcus*, *Bifidobacterium* and *B. fragilis*), the resistance rates of *E. coli* and *Enterococci* were determined by applying the above methods (see