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Evaluation of the safety of primary metabolites of cyadox: Acute and sub-chronic toxicology studies and genotoxicity assessment

Qin Huang [a], Awais Ihsan [d], Pu Guo [b], Xun Luo [b], Guyue Cheng [c], Haihong Hao [c], Dongmei Chen [c], Farrukh Jamil [d], Yanfei Tao [c], Xu Wang [c, **], Zonghui Yuan [a, b, c, *]

[a] National Reference Laboratory of Veterinary Drug Residues (HZAU) and MAO Key Laboratory for Detection of Veterinary Drug Residues, Wuhan, Hubei 430070, China
[b] MOA Laboratory for Risk Assessment of Quality and Safety of Livestock and Poultry Products, Huazhong Agricultural University, Wuhan, Hubei 430070, China
[c] Hubei Collaborative Innovation Center for Animal Nutrition and Feed Safety, Wuhan, Hubei, China
[d] Department of Biosciences, COMSATS Institute of Information Technology, Sahiwal, Pakistan

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ABSTRACT

Cyadox (CYA) is a synthetic antimicrobial agent, belonging to quinoxaline (QdNO) family. Cy1 (bidesoxy cyadox), Cy2 (N4-desoxycyadox) and Cy10 (N1-desoxycyadox) are the primary metabolites of CYA. In our present study, an acute toxicity test, a sub-chronic toxicity test, and a battery of three genotoxicity tests were carried out according to standard protocols. The LD50 of the metabolites were above 5000 mg/kg b.w. The maximum tolerated dose (MTD) of Cy1 and Cy-M (mixture of Cy2 and Cy10) in rats, and the MTD of Cy1, Cy2 and Cy10 in mice were above 6000 mg/kg b.w./day. In subchronic study, rats were separately administered Cy1 and Cy-M at the dose levels of 0, 50, 150 and 2500 mg/kg diet for 90 days, with CYA (2500 mg/kg) as a control. Significant decreases in body weight and changes in clinical serum biochemistry were observed in the high-dose group of Cy1 and Cy-M, as well as CYA. Significant changes in relative weights of organs at 150 and 2500 mg/kg diet of Cy1 and CYA were noted. Additionally, the high-dose groups of Cy1, Cy-M and CYA showed pathological changes near the hepatic portal area. There was no evidence for genotoxic activity of any of the three metabolites in the bacterial reverse mutation test, mouse bone marrow micronucleus assay or an *in vitro* assay for clastogenicity. Based on the sub-chronic study, the target organ of the primary metabolites was the liver, and the no-observed-adverse-effect level for Cy1 and Cy-M was 150 mg/kg diet.

1. Introduction

Quinoxaline-1,4-dioxides (QdNOs) are synthetic antibacterial agents that are used in animal husbandry because of their strong antimicrobial activity (Wang et al., 2015b, 2015a). Cyadox (CYA) is a synthetic quinoxaline derivative (2-formylqui-noxaline-N1,N4-dioxide cyanocetylhydrazone; CAS No. 65884-46-0, $C_{12}H_9N_5O_3$), which is structurally similar to other class members, such as mequindox (MEQ), carbadox (CBX), olaquindox (OLA) and quinocetone (QCT). CYA is very active against *Staphylococcus hyicus*, *Pasterella multocide* and *Escherichia coli* species and also has better growth-enhancing functions in food-producing animals, including fishes, goats, pigs and poultry (Cheng et al., 2004; Ding et al., 2006a, 2006b; Fan et al., 2000; Wang et al., 2005), with less toxic effects than other QdNOs, such as CBX and OLA (Fang et al., 2006; He et al., 2006; Ihsan et al., 2013b; Wang et al., 2011a; Wang et al., 2011b). Therefore, CYA has been regarded as a potential replacement for OLA in China.

Previous studies have shown that OLA, CBX, MEQ and QCT produced clear evidence of genotoxicity in two or more assays while CYA exhibited only weak activity in a bacterial reverse mutation test (Chen et al., 2009; Ihsan et al., 2013a, 2013b). In *in-vivo* studies, the main target organs for QdNOs are liver, kidneys and adrenals (Huang et al., 2009; Ihsan et al., 2010; Wang et al., 2011b, 2010, 2012). CYA was found less toxic than OLA in subchronic,

* Corresponding author. National Reference Laboratory of Veterinary Drug Residues (HZAU) and MAO Key Laboratory for Detection of Veterinary Drug Residues, Wuhan, Hubei 430070, China.
** Corresponding author.
E-mail addresses: wangxu@mail.hzau.edu.cn (X. Wang), yuan5802@mail.hzau.edu.cn (Z. Yuan).

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Abbreviations

8-OhdG	8-hydroxydeoxyguanosine
ALB	albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
AST	aspartate aminotransferase
b.w.	body weight
BUN	blood urea nitrogen
$Ca2^{+}$	calcium
CBX	carbadox
Cl^{-}	chloride
CREA	creatinine
Cy1	bidesoxy CYA
Cy2	N4-desoxycyadox
Cy10	N1-desoxycyadox
CYA	cyadox
DBL	direct bilirubin
DQCT	1,4-bisdesoxyquinocetone
GLP	principles of Good Laboratory Practices
GLU	glucose
FBS	fetal bovine serum
H&E	hematoxylin and eosin
HCT	hematocrit
HGB	hemoglobin
i.v.	intravenously
K+	potassium
M11	2-isoethanol 4-desoxymequindox
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MEQ	mequindox
MIC	minimal inhibitory concentration
MPV	mean platelet volume
MQCA	3-methylquinoxaline-2-carboxylic acid
MTD	maximum tolerated dose
Na^{+}	sodium
NCE	normochromatic erythrocytes
NOAEL	no-observed-adverse-effect level
OLA	olaquindox
P	inorganic phosphorus
PCE	polychromatic erythrocytes
PCT	plateletcrit
PDW	platelet distribution width
PLT	platelet count
p.o.	orally
PT	prothrombin time
PVT	mean platelet volume
QCT	quinocetone
QdNOs	quinoxaline-1,4-dioxides
RBC	red blood cell count
RDW	red cell volume distribution
ROS	reactive oxygen species
TCHOL	total cholesterol
TG	triglyceride
TP	total protein
Urea	blood urea
WBC	white blood cell count

phototoxic and teratogenicity studies (Fang et al., 2006; He et al., 2006; Wang et al., 2011a). In a chronic toxicity study, CYA did not cause tumours under abdomen skin in Wistar rats whereas tumours were observed in three female rats at the 400 mg/kg OLA group at weeks 72, 74 and 75 (Wang et al., 2011b). In the long-term toxicity, CYA (2000 mg/kg diet) produced the same pathological changes in liver when compared with 400 mg/kg OLA group, indicating that CYA presents less toxicity than OLA (Wang et al., 2011b). However, few studies have been carried out to investigate the toxicity of the metabolites of QdNOs, including CYA. It was reported that the metabolites of QCT, *i.e.,* 1,4-bisdesoxyquinocetone (DQCT) and 3-methylquinoxaline-2-carboxylic acid (MQCA), showed less cytotoxicity and genotoxicity than QCT *in vitro* (Zhang et al., 2012, 2014). The N–O reduction metabolite of MEQ, 2-isoethanol 4-desoxymequindox (M11), was detected in the testes following the appearance of 8-hydroxydeoxyguanosine (8-OHdG), which is a marker of oxidative DNA damage (Ihsan et al., 2011). It is usually considered that the metabolites of QdNOs present fewer toxic effects than their parent drugs (Wang et al., 2011c, 2015a). However, the tumorigenic potential of the metabolite of CBX, desoxycarbadox, was apparently greater than that of CBX (WHO, 1991a) indicating that the toxicity of the metabolites should be evaluated carefully. Though many toxicological evaluations of CYA have been carried out both *in vivo* or *in vitro* (Fang et al., 2006; He et al., 2006; Ihsan et al., 2013b; Wang et al., 2011a, 2011b, 2015c), the toxicity of the main metabolites of CYA remains unclear.

The N→O group reduction is one of the main metabolic pathways of CYA (Liu et al., 2009, 2008). In total, twenty-four metabolites of CYA produced by liver microsomes, primary hepatocytes and intestinal microflora systems of rat, chicken and swine have been identified (Wu et al., 2012). Cy1 (bidesoxy CYA), Cy2 (N4-desoxycyadox) and Cy10 (N1-desoxycyadox) are the major and common N→O deoxygenate metabolites of CYA (Wu et al., 2012). Therefore, Cy1, Cy2 and Cy10 were chosen in the present study (Fig. 1).

Due to the wide range and long-term use of CYA in food animals, it is necessary to perform a series of toxicity studies to understand the toxic characters of the main N→O deoxygenate metabolites in accordance with preclinical toxicology guidelines. In the present study, an acute toxicity test, a subchronic toxicity test and a battery of three genotoxicity tests were carried out according to the standard protocols. These data can provide the general toxicity properties, including no-observed-adverse-effect level (NOAEL), target organs and determination of mutagenicity. Furthermore, this data will provide scientific information for further clinical use of CYA and further prove the relationship between the N→O group reduction and QdNOs toxicity.

2. Materials and methods

2.1. Materials

Cyadox (CYA, purity 98%), Cy1 (bidesoxy CYA, purity 98%), Cy2 (N4-desoxycyadox, purity 98.5%), Cy10 (N1-desoxycyadox, purity 98%) and Cy-M [mixture of Cy2 and Cy10, the ratio is 2:1] were obtained from the Institute of Veterinary Pharmaceuticals, Huazhong Agricultural University (Wuhan, PR China). RPMI1640 medium was provided by Hyclone (Shanghai, PR China). Fetal bovine serum (FBS) and newborn calf serum were produced from Hangzhou Sijiqing Biological Materials. (Hangzhou, PR China). Phenobarbital/benzoflavone (10%) induced rat liver S9 was purchased from Platt Bio-Pharmaceutical. (Wuhan, PR China). All other