Regulatory Toxicology and Pharmacology 71 (2015) 585–589

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

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

Safety evaluation of a triazine compound nitromezuril by assessing bacterial reverse mutation, sperm abnormalities, micronucleus and chromosomal aberration

Chenzhong Fei [a], Jie Zhang [b], Yang Lin [c], Xiaoyang Wang [a], Keyu Zhang [a], Lifang Zhang [a], Wenli Zheng [a], Mi Wang [a], Tao Li [a], Sui Xiao [a], Feiqun Xue [a,*], Chunmei Wang [a]

[a] Key Laboratory of Veterinary Drug Safety Evaluation and Residues Research, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, PR China
[b] Chinese Medicine Hospital of Pudong New Area, Shanghai 201200, PR China
[c] Institute of Veterinary Feed Control of Zhengzhou, Henan 450003, PR China

ARTICLE INFO

Article history:
Received 25 August 2014
Available online 30 January 2015

Keywords:
Coccidiosis
Anticoccidials
Nitromezuril
Bacterial reverse mutation (Ames)
Sperm abnormalities
Bone marrow micronucleus
Chromosome aberration

ABSTRACT

Nitromezuril (NZL) is a novel triazine compound that exhibits remarkable anticoccidial activity. However, mutagenicity and genotoxicity of NZL have not been evaluated to date. This study evaluated the potential risks of NZL by testing for bacterial reverse mutation (Ames), mouse sperm abnormality (SA), bone marrow micronucleus (MN) and chromosomal aberration (CA). Mice were orally administered with NZL at 385, 192 and 96 mg/kg, corresponding to 0.5×, 0.25× and 0.125× the LD_{50} of NZL, respectively. No significant increases in SA and CA were found in mice treated with NZL for 5 d and 3 d, respectively ($P > 0.05$). NZL at 96–385 mg/kg did not have significant influence on micronucleated polychromatic erythrocyte counts ($P > 0.05$). These results suggest that NZL is not genotoxic. However, Ames test results were positive both with and without the S9 system for *Salmonella typhimurium* TA98 and TA100, suggesting that NZL may be mutagenic. The mutagenic effects of NZL were different in in vitro and in vivo assays. Further studies should be conducted to confirm the safety of using and developing NZL as a novel anticoccidial drug.

1. Introduction

Coccidiosis is the most important parasitic diseases affecting the poultry industry. Proper control of coccidiosis depends principally on prophylactic chemotherapy with anticoccidial drugs (Peek and Landman, 2011). However, development of drug resistance poses a tremendous challenge, so identification of alternative drugs is an active area of research. Triazine coccidiostats, including diclazuril and toltrazuril, have been widely used in chickens and turkeys because of their remarkable clinical effects on *Eimeria* species. However, they have faced significant problems related to drug resistance in recent years (Conway et al., 2001; Fernandez et al., 2012; Kreiner et al., 2011; Wang et al., 2013).

2-(3-Methyl-4-(4-nitrophenoxy)phenyl)-1,2,4-triazine-3,5(2H, 4H)-dione, also known as nitromezuril (NZL; CAS:1352755-63-5), is a potential novel anticoccidial agent developed in our laboratories (Fei et al., 2013a; Ma et al., 2014). Chemical synthesis of this compound has been described in China Patent No. CN 102285930. The structure of NZL is similar to that of diclazuril and toltrazuril, but no cross-resistance was observed. NZL is effective against different life cycles of various parasites. A previous study reported that the mouse oral LD_{50} of NZL is 769 mg/kg, with a 95% confidence interval of 615–960 mg/kg by the Bliss method and an accumulative coefficient of 4.26 (Fei et al., 2012). A 30 d subacute toxicity test in mice revealed that NZL has no obvious toxic effects (Fei et al., 2013b). A 60 d subchronic toxicity trial in chickens demonstrated that NZL exhibits low toxicity and is safe for clinical use (Fan et al., 2014). However, mutagenicity and genotoxicity of NZL remain unclear to date. Determining the potential risks of NZL is important to establish NZL as a safe drug (Jena et al., 2002; Li, 2004; Merino et al., 2010).

Drugs for registration must be thoroughly tested for safety because of the nominal association between mutagenicity and carcinogenesis. In recent years, many mutagenicity tests with varying degrees of usefulness and extrapolation to humans have been developed. Scientists and regulatory agencies have found that no

* Corresponding author at: No. 518, Ziyue Road, Minhang District, Shanghai 200241, PR China.
E-mail address: fqxue@shvri.ac.cn (F. Xue).

http://dx.doi.org/10.1016/j.yrtph.2015.01.011
0273-2300/

single mutagenicity test can detect all types of potential human mutagens with 100% accuracy or prediction (Hwang and Kim, 2012; Kamath and Rao, 2013). Therefore, most regulatory agencies such as the Food and Drug Administration and the Environmental Protection Agency require a battery of mutagenicity tests, including sperm abnormalities (SA), bacterial reverse mutation (Ames), bone marrow micronucleus (MN) and chromosomal aberration (CA) tests (Kamath and Rao, 2013; Mitchell and Skibinski, 2012).

Comprehensive assessment of mutagenicity and genotoxicity of the novel triazine drug NZL is necessary to understand the risks of potential genotoxicity and its safety for clinical use. In this study, we conducted the Ames test and the mouse SA, MN and CA tests in accordance with the guidelines of veterinary safety evaluation to evaluate the mutagenicity and genotoxicity of NZL and thereby determine its safety for use in dietary supplements.

2. Materials and methods

2.1. Chemicals and animals

NZL (>98%) was synthesized by the Shanghai Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. Sodium carboxy-methyl cellulose (CMC, >98%) was purchased from Aladdin Chemical Co. (Shanghai, China). Cyclophosphamide (CPA, >98%) was obtained from Aesar Chemical Co. (Shanghai, China), while nicotinamide-adenine dinucleotide phosphate (NADP) was bought from Merck (Rahway, NJ, USA). Sodium azide (SA), D-biotin, L-histidine, ampicillin, tetracycline were obtained from Sangon Biotech Co. (Shanghai, China). 2-Amino-anthracene (2AA), 2-amino-fluorene, fenaminosulf, glucose-6-phosphate (G6P), dimethyl sulphoxide (DMSO) and colchicines were obtained from Sigma–Aldrich (St. Louis, MO, USA). Citric acid monohydrate, NaOH, KCl, NaCl, and other chemicals were purchased from Sino-pharm Chemical Reagent Co. Ltd. (Shanghai, China).

Kunming mice weighing 25–30 g were purchased from the Shanghai Laboratory Animal Centre of Chinese Academy of Sciences (License No. SCXK (Hu) 2007-0005). The mice were quarantined in a pathogen-free, well-ventilated room to enable them to acclimatize to their environment. The animals were housed in wire-topped polypropylene cages with rice husk bedding and maintained under standard laboratory conditions at 28 ± 5 °C, 60 ± 5% relative humidity and 12 h photoperiod. The animals were given food in pellet form and water ad libitum.

2.2. Selection of dose

The NZL concentrations used in the Ames test were based on bacterial toxicity estimated in a pre-trial and were as follows: 1000, 200, 40 and 8 μg/plate. The doses selected for in vivo mutagenic and genotoxic assays were set to 385, 192 and 96 mg/kg b.w., corresponding to 0.5×, 0.25× and 0.125× the LD50 of NZL, respectively based on previous toxicity tests and recommended guidelines for toxicity studies of drugs (Kamath and Rao, 2013). Test substances of three concentrations were prepared by serial dilutions using 0.5% CMC. The suspension volumes by oral gavages were approximately 0.2 mL/10 g weight.

2.3. Ames test

Four strains of *Salmonella typhimurium* TA97, TA98, TA100 and TA1535 were kindly given by Dr. Jin Ma, National Shanghai Center for New Drug Safety Evaluation Research Center. Genotypes of these strains were confirmed using the method described by the state standard for Ames (GB 15193.4-2003, PR China). Positive mutagenesis controls were seen in legends of Table 1.

NZL was assayed via the Ames test by standard plate-incorporation assay according to OECD Guideline 471 (1997) and the state standard for Ames (GB 15193.4-2003, PR China). The preliminary Ames test was performed both with and without the S9 activation system. The metabolic activation system (S9 mix) was freshly prepared before each test using an Aroclor-1254-induced rat liver fraction (S-9, MolTox™, Boone, NC, USA). Concentrations of NZL used in this study were 1000, 200, 40 and 8 μg/plate. All of them were diluted in DMSO. Experiments were performed in triplicate. All strains were tested for spontaneous revertant colonies using DMSO as negative (solvent) control. The mutagenic index (MI) was calculated for each dose as the average number of revertants per plate divided by the average number of revertants per plate of the negative (solvent) control. A sample was considered positive when MI was above 2 for at least one of the test doses, and when the response was dose-dependent (Biso et al., 2010).

2.4. SA test

The SA test was carried out according to the veterinary guidelines on SA test in mice and the state standard for sperm abnormalities (GB15193.7-2003, PR China). Fifty male mice were divided into five groups, with 10 mice in each group, using a restricted randomization procedure. Mice in groups 1–3 were treated once daily with 0.5×, 0.25× and 0.125× the LD50 of NZL through oral gavages for five consecutive days. Mice in group 4 (positive control) were administered by gavages with 50 mg/kg b.w. CPA, and mice in group 5 (negative control) received 0.5 mL of normal 0.5% CMC.

At 35 d after the first oral administration, the mice from each dosage group and the controls were sacrificed by cervical dislocation and their epididymis were surgically removed. Sperm smears were prepared from the epididymis as reported by Otubanjo and Mosuro (2001) and described in the guidelines. Slides were randomly coded prior to microscopic analysis, and 1000 sperm cells from each mouse were assessed for morphological abnormalities of the sperm head according to the criteria of Wyrobek and Bruce (1975).

2.5. Bone marrow MN test

The MN test was performed according to the veterinary guidelines on MN test in mice and the state standard for bone marrow cell micronucleus (GB15193.5-2003, PR China). One hundred mice were divided into five groups, with 10 male and 10 female mice in each group, using a restricted randomization procedure. Mice in groups 1–3 were treated twice with 0.5×, 0.25× and 0.125× the LD50 of NZL by oral gavages at 24 h intervals. Mice in group 4 (positive control) were administered with 50 mg/kg b.w. CPA, and mice in group 5 (negative control) received 0.5 mL of normal 0.5% CMC.

At 30 h after the first oral administration, the mice were sacrificed by cervical dislocation. The femur bones of each mouse were separated and cleaned of surrounding muscle tissue. Two ends of the femur were cut until a small opening became visible. Approximately 0.3 mL of bovine albumin was injected into one opening via syringe. The bone marrow was flushed into a clean, dry glass slide, mixed well and smeared onto the slides. Subsequent processing was performed as described by Hwang and Kim (2012). Micronucleus slides were randomly coded prior to microscopic analysis, and 1000 bone marrow polychromatic erythrocytes (PCE) were examined from each mouse. The number of micronucleated PCE, PCE and normochromatic erythrocytes (NCE) was recorded (Kasamoto et al., 2013).