Japanese Journal of Veterinary Research 61(3): 125–128, 2013

INFORMATION

Hokkaido University conferred the degree of Doctor of Philosophy on June 28, 2013 to 3 Recipients.
The titles of theses and other information are as follows:

Evaluation of antineoplastic activity of artemisinin-derived trioxanes in canine tumors

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Artemisinin is a sesquiterpene lactone extracted from Chinese plant *Artemisia annua L*, and is currently used in the treatment of malaria. Through a reaction catalyzed by iron, artemisinin-derived trioxanes are converted to carbon-centered free radicals, which are largely responsible for parasiticidal effect of these chemicals. Recently, artemisinin-derived trioxanes have been found to have antineoplastic properties. The tumoricidal effects are also thought to be mediated by free radical generation. Since iron is a cofactor in the synthesis of deoxyriboses, most neoplastic cells overexpress cell surface transferrin receptors and have greater intracellular iron concentrations than normal somatic cells.

The aim of this study was to investigate the antineoplastic effect of artemisinin-derivatives in canine tumors. In the first part of the study, the clinical adverse effects and potential antitumor effects of orally administered artemisinin were investigated in dogs with spontaneously occurring tumors. The toxicity was minimal, although objective response was observed only in one dog, suggesting that derivatives with higher potency should be considered for further study. In the second part of the study, the mechanism of cytotoxicity of dihydroartemisinin was investigated using canine osteosarcoma cell lines. The *in vitro* cytotoxic effect of dihydroartemisinin was demonstrated in all four canine osteosarcoma cell lines. Dihydroartemisinin induced concentration-dependent free radical generation by an iron-dependent mechanism, resulting in growth inhibition in all four cell lines tested. Dihydroartemisinin also induced cellular apoptosis and G_2/M cell cycle arrest in a concentration-dependent manner. In the third part of the study, the correlation between the cytotoxicity of dihydroartemisinin and intracellular iron concentration was investigated in two canine histiocytic sarcoma cell lines. The cytotoxicity of dihydroartemisinin was enhanced by increasing the cellular iron concentration and inhibited by chelating iron from the culture media. These findings lay basis for further research of artemisinin-derived trioxanes in treatment of malignant tumors in dogs.

The original papers of this thesis appeared in *Am. J. Vet. Res.*, **69**: 519–526 (2008) and *J. Am. Anim. Hosp. Assoc.*, in press.

Effects of furazolidone and its metabolites on hepatic drug metabolizing enzymes in rat and chicken

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The nitrofuran antimicrobial drug, N-(5-nitro-2-furfurylidene)-3-amino-2- oxazolidone (furazolidone; FZ) has been used for treatments of certain bacterial and protozoal infections in human and animals for $>$ 40 years. The use of FZ in food-producing animals has been forbidden in European Union (EU) countries, the USA, Japan as well as many other countries owing to its mutagenic and carcinogenic activities. However, FZ is still in use in some countries of the Middle and Far Eeast as a feed additive for livestock and shrimp. It is also used to treat infectious diseases in humans, especially for eradication of *Helicobacter pylori*. It is known that the injection of FZ causes alterations of the expression levels of phase I drug-metabolizing enzymes (cytochrome P450, CYP), which participate in the phase I metabolism of numerous drugs in experimental animals. There is, however, little investigation of the effect of FZ on the CYP-related activity especially in rat and chicken. The aim of present studies was to investigate the effect of successive bolus doses of FZ and its metabolites, AOZ and HEH in some studies, on CYP-related activities in rat and chicken livers.

In this study, FZ (125 mg/kg) was orally administered to Wistar rats for 3 days. Results of the Ames test using the S-9 fraction of rats treated with FZ showed a significant increase of the number of revertant colonies. Western blot analysis of hepatic CYP isozymes induced by FZ, revealed a remarkable induction of CYP1A1 apoprotein, but CYP1A2 and CYP2E1 apoproteins were not altered. In addtiton, the expression level of CYP1A1 mRNA in rats was significantly enhanced by FZ treatment. We concluded that FZ is apparently mutagenic and induces transcription of the CYP1A1, which metabolically activates numerous promutagens, in hepatocytes.

Next, the present study aimed to investigate the effects of successive bolus doses of FZ and its metabolite 3-amino-2-oxazolidinone (AOZ) on CYP-related activities in the livers of rats and chickens. Female Wistar rats and White Leghorn chickens were orally administered with FZ once a day for 4 consecutive days. FZ-treated chickens showed an increase of multiple CYP-related activities, however, rats treated with FZ did not show these changes. In chickens, treatment with FZ also induced production of microsomal CYP2C6-like apoprotein. The present study demonstrated that FZ caused a multiple-type induction of CYP-related activities in chickens, but not in rats.

Finally, the present study aimed to show the effect of FZ on the activity of microsomal enzymes that metabolize FZ, and to identify the enzyme that contributes to FZ metabolism in chickens. Wistar rats and White Leghorn chickens were administered FZ once a day for four consecutive days. FZ metabolism was accelerated by FZ administration in chickens, but not in rats. The elevation of FZ metabolism coincided with the induction of NADPH cytochrome P450 reductase (CPR) activity in chickens, but such induction was not observed in rats. FZ metabolizing activities were inhibited in the presence of CPR inhibitor (dephenylene iodonium chloride) but not by the addition of archetypal cytochrome P450 inhibitors (CO or n-octylamine). The present study concluded that FZ accelerated its own metabolism in the chicken

by induction of the activity of CPR.

In conclusion, it was elucidated that FZ treatment in chicken causes induction of drug-metabolizing enzymes observed previously in rats and pigs. FZ may therefore induce multiple CYPs in humans and numerous animal species. Compounds, including FZ, which induce multiple CYP isozymes, have various toxic actions in animals, such as increasing the toxic effect of some drugs and pollutants. Therefore, more attention should be paid to the usage of FZ.

The original papers of this thesis appeared in *J. Vet. Med. Sci.*, **56**: 667–670 (1994), *J. Vet. Med. Sci.*, **70**: 223–226 (2008) and *Pestic. Biochem. Phys.*, **100**: 135–139 (2011).

Characterization of drug-resistant *Mycobacterium tuberculosis* strains isolated in Nepal

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Tuberculosis (TB) is a major public health problem in Nepal. The situation is further worsened with the emergence of multidrug-resistant tuberculosis (MDR-TB; TB caused by *Mycobacterium tuberculosis* strains resistant to more than two drugs including isoniazid (INH) and rifampicin (RIF)) and extensively drug-resistant tuberculosis (XDR-TB; TB caused by *M. tuberculosis* strains resistant to more than four drugs including INH, RIF, one of fluoroquinolones and any of the second-line anti-TB injectable drugs). However, information on drug-resistant associated mutations in MDR- and XDR-*M. tuberculosis* isolates from Nepal was lacking before our study.

As a part of my PhD work, I investigated the type and frequency of drug resistance-conferring mutations among MDR-*M. tuberculosis* isolates from Nepal. Mutations affecting the 81-bp RIF resistance-determining region (RRDR) of *rpoB* were identified in 106 of 109 (97.3%) RIF-resistant isolates. Codons 531, 526, and 516 were the most commonly affected, at percentages of 58.7, 15.6, and 15.6, respectively. Of 113 INH-resistant isolates, 99 (87.6%) had mutations in the *katG* gene, with Ser315Thr being the most prevalent (81.4%) substitution. Mutations in the *inhA* promoter region were detected in 14 (12.4%) INH-resistant isolates; 12 of which had mutation at position -15 in the *inh*A promoter. No mutation was detected in 2.8% RIF-resistant and 6.2% of INH-resistant isolates.

Furthermore, 13 XDR-*M. tuberculosis* isolates were detected among 109 MDR isolates. Mutations predominant among XDR-TB were Ser531Leu in *rpo*B gene (92.3%), Ser315Thr in *katG* gene (92.3%), Asp94Gly in *gyr*A gene (53.9%) and A1400G in *rrs* gene (61.5%). Spoligotyping and multilocus sequence typing of these isolates revealed 69% belonged to Beijing family, especially modern type. Infections of this family were more common among younger generation than those belonging to other spoligotype families. Further typing with 26-loci variable number of tandem repeats analysis suggested current spread of Beijing genotype XDR-*M. tuberculosis* among people in Nepal. The transmission of XDR-TB was speculated not only within a city but also between two cities, apart more than 650 Km.

In conclusion, our study provides valuable information on molecular mechanism of drug resistance in MDR- and XDR-*M. tuberculosis* isolates from Nepal. It can serve as a basis for developing or improving rapid molecular drug-susceptibility tests to monitor drug-resistant isolates. Additionally, genotypic data suggested the possible transmission of XDR-*M. tuberculosis* strains in Nepal that highlights an urgent need to identify patients suffering from this incurable disease and treat those patients in isolated wards to prevent further spread to the community, and to reinforce the TB policy with regard to control and detection strategies.

The original papers of this thesis appeared in *Antimicrob. Agents Chemother.*, **56**: 2831–2836 (2012) and *Tuberculosis*, **93**: 84–88 (2012).