



Title	INFORMATION: Theses for the Doctor of Veterinary Medicine (equivalent to Ph.D.)
Citation	Japanese Journal of Veterinary Reseach, 54(2-3), 141-144
Issue Date	2006-11-30
Doc URL	<a href="http://hdl.handle.net/2115/16910">http://hdl.handle.net/2115/16910</a>
Type	bulletin (article)
File Information	JJVR54-2-3-6.pdf



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## INFORMATION

Hokkaido University conferred the degree of Doctor of Philosophy (Ph.D) in Veterinary Medicine on June 30, 2006 to 2 recipients and September 25, 2006 to 1 recipient.

The titles of theses and other information are as follows :

### Basic and clinical studies on photodynamic therapy using benzoporphyrin derivative monoacid ring A (BPD-MA) for animal tumor

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Photodynamic therapy (PDT) involves the administration of a tumor-localizing photosensitizer that is followed by light activation. To date, the intracellular localization of photosensitizers as well as the mechanisms of cellular responses associated with PDT is being discussed. In this study, benzoporphyrin derivative monoacid ring A (BPD-MA), which is a second generation photosensitizer, was used.

The aim of the first part of this study was to investigate the intracellular localization and concentrations as well as the photodynamic effects of BPD-MA in four types of rodent tumor cells, including mouse pigmented melanoma, B16F1 ; mouse pulmonary squamous cell carcinoma, KLN205 ; mouse osteosarcoma, LM8 ; and rat glioma, RG2. A good correlation was observed between cell survival at 0.10 µg/ml of BPD-MA and sensitizer uptake/ $1 \times 10^6$  cells ( $r = -0.99$ ) or the plating efficiency of cells ( $r = 0.99$ ). Phototoxicity was found to be dependent on BPD-MA dose or fluence. At 3-hours after the irradiation, a significant difference was observed in the proportion of apoptotic cells among the four types of rodent tumor cells ( $p = 0.024$ ). These cells also showed inherent differences

in photosensitivity. In conclusion, cell responses to PDT depend on several factors such as tumor cell lines, photosensitizer dose, and fluence.

The aim of the second part of this study was to assess the antitumor effect and tumor blood flow dynamics after cellular-targeting PDT or vascular-targeting PDT using BPD-MA in both KLN205 and LM8 tumors. In both these tumors, the vascular-targeting PDT was more effective in inducing the tumor growth delay than the cellular-targeting PDT. Based on the power Doppler ultrasound examination, as compared to the cellular-targeting PDT, the vascular-targeting PDT showed a more significant decrease in the vascularity and the blood perfusion. It was consequently showed that the vascular-targeting PDT enhanced the vascular shutdown effect and the antitumor effect by damaging the tumor vasculature (antiangiogenic PDT).

The aim of the third part of this study was to investigate the clinical pharmacokinetics of antiangiogenic PDT using BPD-MA in dogs with head tumors. After intravenously injecting BPD-MA at a dose of 0.5 mg/kg, its mean half-life was found to be  $8.14 \pm 5.34$  h ; mean clearance,  $35.13 \pm 9.62$  ml/(h · kg) ;

mean distribution volume,  $0.08 \pm 0.01$  l/kg ; and mean steady state distribution volume,  $0.38 \pm 0.311$ /kg. By considering the pharmacokinetic parameters of BPD-MA, antiangiogenic PDT using BPD-MA could be completed within a short time period and be performed repeatedly because BPD-MA can be rapidly cleared from the tissues, and hence, shows low accumulation.

Finally, the aim of this study was to assess the efficacy of antiangiogenic PDT using BPD-MA in 19 dogs with head tumors. Each tumor was irradiated at 15 minutes after initiating the intravenous infusion of 0.5 mg/kg BPD-MA. In cases that were followed-up for more than 1 year after PDT, the median survival time of 6 dogs with oral tumors was found to be 423 days (range, 300-743 days) and a 1-year survival rate was observed in 67% of the dogs. In 5 dogs with nasal cavity tumors, the values were 533 days (range, 129 - 694

days) and 60%, respectively. The computed tomography (CT) enhancement values before and after PDT were significantly different ( $p < 0.001$ ) and were  $54.3 \pm 25.8$  Hounsfield units (HU) (21 treatments in 15 dogs) and  $5.5 \pm 5.7$  HU (18 treatments in 11 dogs). The mean CT enhancement value of the tumors in which 19 treatments were effective was 57.2 HU and that for tumors in which 2 treatments were ineffective was 27.5 HU. The major side effect was temporary edema around the treated area for 1 week after PDT ; however, it did not require any specific treatment. Antiangiogenic PDT might be a promising method for treating canine solid malignant tumors without causing any serious side effects. Angiographic CT might play a useful role in selecting antiangiogenic PDT cases and in determining the therapeutic effect after antiangiogenic PDT.

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The original papers of this thesis appeared in *Cancer Lett.*, 243 : 281-292 (2006), *J. Vet. Med. A*, 53 : 108-112 (2006), and *Cancer Lett.*, in press (2006).

### Development of new typing methods of *Actinobacillus pleuropneumoniae* based on the genetic diversity of the protective outer membrane lipoprotein

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*Actinobacillus pleuropneumoniae* is a causative agent of porcine pleuropneumonia characterized by a fibrinous-hemorrhagic pneumonia. The disease occurs worldwide and results in serious economic losses to the pig-rearing industry. In this study, new typing methods based on the genetic diversity of an outer membrane lipoprotein have been developed for control of porcine pleuropneumonia

caused by the organism.

First, the outer membrane lipoprotein and its gene from an *A. pleuropneumoniae* field isolate (serotype 5a) have been characterized as a candidate gene and protein for typing of the organism. Consequently, the outer membrane protein has been shown to be a protective antigen of the organism. Furthermore, Southern blot analyses using the gene

as a probe have shown the presence of DNA sequence, homologous to the gene for the outer membrane lipoprotein of the field isolate (serotype 5a), in *A. pleuropneumoniae* reference strains (serotypes 1 to 12). Second, the nucleotide sequence of the gene for the outer membrane lipoprotein of *A. pleuropneumoniae* reference strains (serotypes 1 to 12) has been determined. Comparison of the nucleotide sequence of the gene revealed diversity of the gene for the outer membrane lipoprotein.

Based on these results, a new DNA-based typing method (PCR-Restriction Fragment Length Polymorphism (RFLP) typing method) using the diversity of the gene for the outer membrane lipoprotein has been developed. This method can divide *A. pleuropneumoniae* reference strains (serotypes 1 to 12) and field isolates (serotypes 1, 2, 3, 5, 7 and 10, n=42) into five groups.

Finally, a new serotyping method using Western blot analysis with specific antisera

against three antigenically distinct outer membrane lipoproteins has been developed. This method revealed antigenic diversity of the outer membrane lipoprotein and can differentiate reference strains of *A. pleuropneumoniae* serotypes 1, 2, 4, 5a, 5b, 6, 7, 8, 9, 10 and 11 into three groups. The result of grouping by the Western blot analysis was correlated with that of grouping by the PCR-RFLP typing method described above.

The typing methods developed in this study are new methods based on a particular gene and its encoding protective protein, which are different from the currently used typing method using polyclonal antisera raised against whole bacterial cells of *A. pleuropneumoniae*. Typing of protective antigens of *A. pleuropneumoniae* is very important for choice of vaccine strains. The new typing methods developed in this study should be used as tools which are effective and practical for control of porcine pleuropneumonia caused by *A. pleuropneumoniae*.

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The original papers of this thesis appeared in *Microb. Pathog.*, 18 : 29-36 (1995), *J. Vet. Med. Sci.*, 59 : 213-215 (1997), and *FEMS Microbiol. Let.*, 167 : 303-308 (1998).

## Lipid rafts are essential for calcium-dependent apoptosis induced by 2-chloro-2'-deoxyadenosine (Cladribine)

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2-Chloro-2'-deoxyadenosine (2CdA; Cladribine) is a purine nucleoside analog that is cytotoxic to leukemia cells. It induces apoptosis through the overexpression of death receptors Fas and DR5 and the formation of death-inducing signaling complex (DISC) in the manner of ligand-independent self activa-

tion of death receptors followed by caspase-3 activation in MOLT-4 cells.

Recently, ganglioside- and cholesterol-enriched membrane microdomains (lipid rafts) were proposed to function as platforms for receptor signaling. To investigate whether lipid rafts play a crucial role in 2CdA-induced

apoptosis, lipid rafts on the cell surface of 2CdA-treated cells were evaluated by flow cytometry and the fluorescent microscopic observation using FITC-CTx, which serves as a marker for the presence of lipid rafts on the cell surface. It was indicated that 2CdA induced GM1 expression and the formation of lipid rafts on the cell surface in time-dependent manner. Lipid rafts can be isolated in the nonionic detergent Triton X-100 based on their insolubility and recovered at the low density position of sucrose gradients. Sucrose density gradient centrifugation showed that overexpression of Fas and DR5 induced 2CdA were translocated in lipid rafts. To confirm whether the crucial role of lipid rafts of 2CdA-induced apoptosis was generally observed in the other leukemia cells, the effects of disruption of lipid rafts by M $\beta$ CD and filipin on 2CdA-induced apoptosis were investigated in four human acute lymphoblastic leukemia (ALL) cell lines comprised of T cells (MOLT-4 and Jurkat) and B cells (NALM and BALL-1). M $\beta$ CD and filipin significantly inhibited 2CdA-induced apoptosis in these cells, indicating the crucial role of lipid rafts in the induction of apoptosis in leukemia cells.

Since 2CdA is transported into the cell by nucleoside transporters on the plasma membrane, then converted to its triphosphate form, 2CdATP, and incorporated into DNA to inhibit DNA synthesis, the contribution of

lipid rafts to the cellular uptake of 2CdA was investigated. Neither M $\beta$ CD nor filipin had any effect on the cellular uptake of 2CdA, the incorporation into DNA or the inhibition of DNA synthesis. Furthermore, M $\beta$ CD and filipin had no effects on 2CdA-induced activation of caspase-3. These results suggest that lipid rafts regulate the events of downstream of caspase-3 activation.

The treatment of 2CdA induced elevation of the intracellular calcium concentration, which was partly inhibited by M $\beta$ CD and filipin. This suggests that the rafts are partly important for the entry of extracellular calcium in 2CdA-treated MOLT-4 cells. Furthermore, 2CdA-induced apoptosis was partly inhibited by the Ca<sup>2+</sup> chelators BAPTA-AM and EGTA. In addition, the L-type Ca<sup>2+</sup> channel blocker nifedipine also partly inhibited it. These results demonstrate that lipid rafts play a crucial role in death receptor-dependent apoptosis in 2CdA-treated MOLT-4 cells by regulating calcium entry.

These results indicate that lipid rafts partly contribute to 2CdA-induced apoptosis by regulating Ca<sup>2+</sup> influx via the plasma membrane in MOLT-4 cells. The finding of this study indicates the new mechanism in anticancer drug-induced apoptosis contributed to lipid rafts and presents the essential information for development of new anticancer drugs.