

# Potential Growth Inhibitory Effect of Maitake D-Fraction on Canine Cancer Cells

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## CLINICAL RELEVANCE

The postulated anticancer effect of D-fraction, the bioactive extract of the maitake mushroom, on three types of canine cancer cells (CF33, CF21, and CL-1) was evaluated. The effect of D-fraction on several human cancer cells was also investigated. The effect of other  $\beta$ -glucan products was likewise examined. D-fraction was highly effective on the canine cancer cells, either potently inhibiting cell growth or directly killing cells. Similar effects were also demonstrated in certain human cancer cells. However, other  $\beta$ -glucan products had no such effects on canine cancer cells. Therefore, D-fraction is a potent natural agent that could be useful in treating canine as well as other veterinary cancers.

## ■ INTRODUCTION

Maitake (*Grifola frondosa*) is an edible, tasty mushroom that has been praised and beloved by Japanese people for hundreds of years. This mushroom has been available by cultivation since the mid-1980s, allowing scientists to study its medicinal properties as claimed in anecdotes and folklore. Numerous physiologic benefits of maitake have been postulated, ranging from antitumor effects to treatment for hypertension, diabetes, hypercholesterolemia, obesity, and hepatitis B infection.<sup>1-8</sup> Maitake's antiviral activity against HIV infection (AIDS) was also confirmed by the National Cancer Institute in 1992.<sup>9</sup>

Most research on maitake has been performed using the bioactive extract named D-fraction (Maitake D-Fraction, Maitake Products, Inc., Ridgefield Park, NJ) to assess its

potential efficacy on various human malignancies. D-fraction is the protein-bound polysaccharide known as  $\beta$ -glucan, which has a molecular weight of approximately  $1 \times 10^6$  D and consists of either  $\beta$ -1,6-linked glucan with  $\beta$ -1,3 branches or  $\beta$ -1,3 glucan branched with  $\beta$ -1,6 glucosides.<sup>1,10</sup> Of all the extracted fractions, D-fraction had the most potent antitumor activity in tumor-bearing mice, resulting in a profound reduction in cancer proliferation.<sup>11</sup> It is interesting to note that D-fraction was capable of promoting tumor suppression/regression in these mice regardless of whether the route of administration was oral, intravenous, or intraperitoneal,<sup>11,12</sup> because many similar mushroom products have been known to be less effective when given orally.

It is also important to address the safety of D-fraction, which is a critical and substantial

issue. An early animal study (using mice) and a nonrandomized clinical study on human patients with various cancers confirmed the safety of D-fraction without any adverse effects.<sup>13,14</sup> This was further acknowledged by the fact that the FDA exempted D-fraction from a phase I study of toxicology. The FDA then granted an Investigational New Drug (IND) application to Maitake Products to conduct a phase II pilot study using D-fraction on patients with ad-

any serious diseases. However, such studies and data are currently lacking or are insufficient to adequately address the efficacy of those supplements on dogs. More basic scientific and clinical studies should be actively conducted on dogs and other domestic animals to build up such a database.

Accordingly, I examined whether a newly developed D-fraction for veterinary use (Grifron-Pet DVM Fraction, Maitake Prod-

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***A phase II pilot study using D-fraction on patients with advanced breast and prostate cancer is currently under way at several institutions and hospitals.***

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vanced breast and prostate cancer,<sup>15</sup> which is currently under way at several institutions and hospitals.

It has recently been reported that D-fraction was capable of inducing apoptosis (programmed cell death) in human prostatic cancer cells in vitro.<sup>16</sup> A separate study further revealed a chemosensitizing effect of maitake; the cytotoxic activity of the anticancer agent carmustine was significantly enhanced in combination with a nontoxic concentration of D-fraction, resulting in approximately 90% cell death.<sup>17</sup>

These studies then led me to explore a potential anticancer effect of D-fraction on canine cancers. The dog is a close companion of humans and is exposed to the same environmental factors and influences as humans. Like humans, dogs also suffer from various types of cancers, requiring timely and proper treatments. Although many treatment options for dogs are available, the efficacy of these treatments has not yet been established. In addition, various nutritional supplements can be given to dogs to maintain their general health and possibly to prevent them from developing

ucts) might have a growth regulatory effect, assessed by cell number and viability, on common canine cancer cells, including lymphoma, mammary gland cancer, and connective tissue cancer. The same study was also performed on human cancer cells to test whether D-fraction could effectively overcome a species barrier. Furthermore, an additional study was conducted to compare the efficacy of D-fraction with that of other commercially available mushroom products on canine cancer cells. The hypothesis was that D-fraction would negatively impact cell growth and/or viability of canine and human cancer cells while other mushroom products would have no effect on cell growth and viability.

## ■ MATERIALS AND METHODS

### Cell Culture

Canine mammary gland cancer cells (CF33) and connective tissue cancer cells (CF21) were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Canine lymphoma cells (CL-1)<sup>18</sup> were kindly given as a gift from Hajime Tsujimoto, DVM, at The University of Tokyo (Department of Veterinary

Internal Medicine, Faculty of Agriculture, Tokyo). Both CF33 and CF21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM); CL-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml). These cells were maintained at 37°C in a humidified incubator in an atmosphere of 95% air and 5% carbon dioxide. The medium was routinely changed every 3 days, and cells were passaged or split weekly via trypsinization. In addition, several "human" cancer cells were maintained for separate experiments, including leukemia (HL-60), gastric (AGS), breast (MCF-7), bladder (T24), kidney (ACHN), liver (HepG2), lung (A549), and brain (U-87) cancer cells. They were cultured in specific media (RPMI-1640 [for HL-60], Ham's F-12 [for AGS and A549], McCoy's 5a modified [for T24], and Minimum Essential Eagle [for MCF-7, ACHN, HepG2, and U-87]) with 10% FBS and antibiotics. All these cells were then routinely subcultured as described above.

### Experiments

After confluency was attained (7 days), CF33, CF21, and CL-1 cells were seeded at the initial cell density of  $1 \times 10^5$  cells/ml in six-

well culture plates and were cultured with varying concentrations (0, 265, 530, 1,060, and 1,600 µg/ml) of D-fraction. Cell morphology or appearance was also monitored every day under a microscope. No medium was changed during the experiment, and cell numbers and viability were assessed at 4 days as described

below. Similarly, eight types of human cancer cells were also subjected to a dose-dependent study of D-fraction to ascertain its potential effects on cell numbers and viability of other cancer cells from species other than dogs. Such experiments essentially followed the same protocol for the above canine cancer study. In addition, it was of interest to examine whether other mushroom products might have similar or any effects, compared with D-fraction, on canine cancer cells. Those products included ASC (mixed powder of *Agaricus blazei* mushroom and shark cartilage), MSK (mixed powder of three mushrooms and three herbs), and AHC (extract from mycelia of several mushrooms). CF33, CF21, and CL-1 cells were cultured with the varying concentrations (0–1,000 µg/ml) of ASC, MSK, or AHC, and cell numbers and viability were determined at 4 days. All experiments were performed in triplicate.

### Cell Count and Cell Viability Assessment

The trypan blue exclusion test was performed to determine cell numbers and viability. Control or agent-treated cells (in 2-ml culture volume) were trypsinized at specified times by 200 µl trypsin-EDTA solution (Sigma, St. Louis, MO), and cell suspension was obtained by neutralizing trypsin with 800 µl

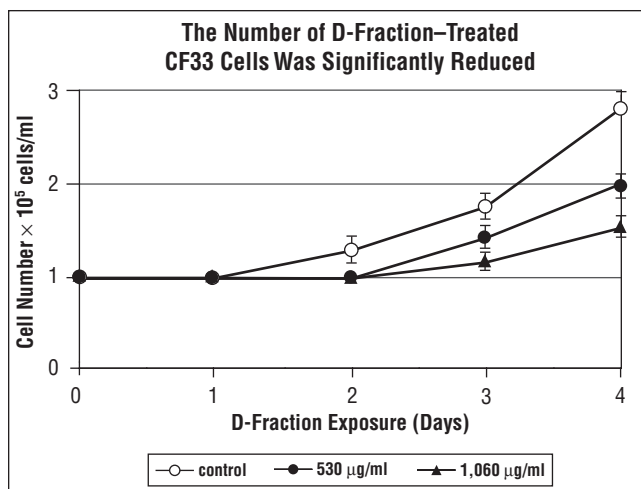
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## *The hypothesis was that D-fraction would negatively impact cell growth and/or viability of canine and human cancer cells.*

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fresh medium (i.e., a total of 1 ml cell suspension). A quantity of 200 µl of each cell suspension was removed and mixed with an equal amount of 0.4% trypan blue solution (Sigma). After incubation at room temperature for 5 minutes, the number of "unstained" viable cells (excluding trypan blue) was counted us-

well culture plates and were cultured with varying concentrations (0, 265, 530, 1,060, and 1,600 µg/ml) of D-fraction. Cell morphology or appearance was also monitored every day under a microscope. No medium was changed during the experiment, and cell numbers and viability were assessed at 4 days as described



**Figure 1.** Effect of D-fraction on the number of CF33 cells. Canine cancer CF33 cells were cultured with either 530 or 1,060 µg/ml of D-fraction for 1, 2, 3, or 4 days. Each test was run in triplicate. At indicated times, cell numbers and viability were determined as described in Materials and Methods. Cell numbers in each condition were then calculated as mean ± SD and plotted against days. Cell viability was nearly 100% in all tests.

ing a hemacytometer to estimate cell count and viability. Cell count was repeated three times for each sample.

Although the trypan blue exclusion test is widely accepted for assessing cell viability, I routinely performed an additional cell viability test for confirmation. The alamarBlue cell viability test (Biosource International, Camarillo, CA) has been described as a better way to assess cell viability because it can be quantified by its optical density or fluorescence for greater sensitivity.<sup>19</sup> AlamarBlue is a nontoxic, nonradioactive redox (metabolic) dye; its oxidized state is blue and nonfluorescent. When it is taken up by viable cells, the intracellular metabolic reactions convert (reduce) it to a red form with high fluorescence. These changes in color and fluorescence are shown to be proportional to the number of viable cells. Briefly, 100 µl of cell suspension described above was added to a flat-

bottomed 96-well culture plate, followed by the addition of 20 µl of alamarBlue; the plate was then placed in an incubator (at 37°C and 5% carbon dioxide) for 1 hour. The plate was allowed to cool to room temperature for 10 minutes, and the fluorescence was read using a 96-well fluorometer with excitation at 530 nm and emission at 590 nm. Background fluorescence measurements were determined from those containing only alamarBlue in culture medium without cells. All samples were assayed in triplicate, and fluorescence readings were corrected for background.

### Statistical Analysis

All data are presented as mean ± SD, and statistical differences between groups were assessed with the unpaired Student's *t*-test. A value of *P* < .05 was considered significant.

## RESULTS

### Effects of D-Fraction on Three Canine Cancer Cells

A pilot study investigating the dosage effects of D-fraction (0, 265, 530, 1,060, and 1,600 µg/ml) on CF33 and CF21 cells indicated two effective concentrations (530 and 1,060 µg/ml), with an optimal duration of 4 days. Concentrations less than 265 µg/ml had no effect, but 1,600 µg/ml was highly cytotoxic, killing more than 95% of cells. Accordingly, the time-dependent cellular effects of these specific concentrations were examined. Compared with control (untreated) CF33 cells, the number of D-fraction-treated cells was significantly (*P* < .05) lower, resulting in approximately 45% and 70% reduction in cell num-

**TABLE 1. Effects of D-Fraction on the Number of Canine Cancer Cells**

<i>D-Fraction Concentration (µg/ml)</i>	<i>Cell Numbers (% of Control)<sup>a</sup></i>		
	<i>CF33 (4 days)</i>	<i>CF21 (4 days)</i>	<i>CL-1 (24 hours)</i>
0 (control)	100%	100%	100%
530	≈55% (≈45% ↓)	≈65% (≈35% ↓)	≈80% (≈20% ↓) <sup>b</sup>
1,060	≈30% (≈70% ↓)	≈25% (≈75% ↓)	<10% (>90% ↓) <sup>b</sup>

<sup>a</sup>Cell count was performed in triplicate for each cell line. Cell numbers are expressed as percentages of controls (100%). The values in parentheses also indicate the percent reduction in cell numbers relative to controls.

<sup>b</sup>The reduction in cell number was primarily a result of cell death (loss of cell viability), confirmed by the alamarBlue cell viability test.

bers with 530 and 1,060 µg/ml D-fraction, respectively, in 4 days (Figure 1). However, nearly all cells (>98%) were found to be viable, indicating that such reduced cell numbers without cell death more likely reflects growth attenuation or inhibition, because cell growth could be defined by cell number and cell viability. Principally similar results were seen in a time-dependent study of CF21 cells as well (data not shown). These results of both CF33 and CF21 cells are summarized in Table 1. In addition, no drastic morphologic changes were

cells actually lost plasma membrane integrity, becoming extensively fragmented (dead cells) by 24 hours. Such a rapid, drastic reduction in (viable) cell numbers as a result of cell death is likely indicative of a “cytotoxic” effect of D-fraction on CL-1 cells.

#### **Effects of D-Fraction on Human Cancer Cells**

It was of interest to ascertain the versatility of D-fraction or whether D-fraction may affect other cancer cells from species other than the

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detected in either cell line with D-fraction treatment. However, many cells subsequently became detached from the plates (loss of cell adherence), indicating a cessation of cell growth in typical monolayer cultures.

In contrast, CL-1 cells appeared to be more susceptible to D-fraction, because approximately 20% and more than 90% of these cells were “dead” with 530 and 1,060 µg/ml of D-fraction, respectively, within 24 hours (Table 1). Morphologically, those D-fraction-treated

dog. Its dose-dependent effects were examined on eight human cancer cells as described in Materials and Methods. No effects on AGS, ACHN, and A549 cells were detected at the given concentrations of D-fraction, even after 4 days; slightly (<10%) reduced cell numbers were seen in MCF-7 and HepG2 cells, indicating some growth inhibition (Table 2). D-fraction showed significant effects on HL-60, T24, and U-87 cells. Approximately 50% and 90% of HL-60 cells were “dead” with 265 and 530

**TABLE 2. Effects of D-Fraction on Cell Number and Growth of Human Cancer Cells**

<i>Cancer Cell</i>	<i>Effects on Cell Number/Growth</i>
HL-60 (leukemia)	≈50% cell death with 265 µg/ml D-fraction in 24 h ≈90% cell death with 530 µg/ml D-fraction in 24 h
AGS (gastric)	No effect
MCF-7 (breast)	Insignificant reduction (<10%) in cell number/growth
T24 (bladder)	≈50% cell death with 530 µg/ml D-fraction in 24 h >95% cell death with 1,060 µg/ml D-fraction in 24 h
ACHN (kidney)	No effect
HepG2 (liver)	Insignificant reduction (<10%) in cell number/growth
A549 (lung)	No effect
U-87 (brain)	≈90% reduction in cell number/growth with 265 µg/ml D-fraction in 3 days

µg/ml D-fraction, respectively, within 24 hours. Nearly 50% and over 95% of T24 cells were also “dead” with 530 and 1,060 µg/ml D-fraction, respectively, in 24 hours. Although it required 3 days, there was an approximately 90% reduction in the number of U-87 cells (without cell death) with only 265 µg/ml D-fraction. Overall, D-fraction demonstrated its growth inhibitory or cytotoxic effects on three of eight human cancer cells tested; an insignificant (<10%) growth reduction was seen on two cell lines, and no effect was seen on three cell lines.

**Effects of Other Mushroom Products on Canine Cancer Cells**

Since D-fraction thus far seems to generally work on both human and canine cancer cells, I also examined whether other mushroom products for veterinary use (ASC, MSK, and AHC) might have similar effects on canine cancer cells, as described in Materials and Methods. Compared with D-fraction, none of the products tested at given concentrations, including 500 and 1,000 µg/ml, had any significant effects on the number of CF33, CF21, or CL-1 cancer cells.

**DISCUSSION**

In the present study, the primary objective was to obtain useful information regarding the effect of maitake D-fraction on the growth of canine cancer cells (CF21, CF33, and CL-1) in vitro, by assessing changes (increases or decreases) in cell numbers along with cell viability following D-fraction treatment. It is critical to accurately determine cell numbers and viability because these two factors would indicate the actual status of proliferative activity or cell growth. It is also true that there are several other ways to assess cell proliferation in terms of DNA synthesis, mitotic process, or metabolic analysis. For example, commonly applied methods for assessing or measuring cell proliferation include the use of radiolabeled biochemicals (e.g., [<sup>3</sup>H]-thymidine), tetrazolium compounds (e.g., 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [MTT]), propidium iodide (PI) staining, bromodeoxyuridine incorporation, and Hoechst 33258 (bisbenzimidazole H33258) staining.

In fact, I performed a pilot study using [<sup>3</sup>H]-thymidine incorporation and PI staining to assess cell growth by specifically measuring DNA synthesis during cell division. Such results were

principally comparable to those obtained by trypan blue and alamarBlue tests assessing cell numbers and viability. Avoiding the use of a hazardous, radioactive [ $^3\text{H}$ ]-thymidine or cutting down the operational cost of a flow cytometer (for PI staining), I employed the trypan blue and alamarBlue tests instead. Both tests are being widely and routinely used because they are simple, highly reproducible, less toxic, and nonradioactive. I defined *cell growth* as “changes in cell numbers without cell death, which are indicative of a growth inhibition or promotion.” For instance, a reduction in cell numbers indicates growth inhibition when those cells are all viable. In contrast, when the reduced cell numbers are primarily attributable to cell death, it more likely results from a cytotoxic (not a growth inhibitory) effect because

concentrations of D-fraction in animals, and this question certainly needs to be adequately answered. Nevertheless, the effectiveness of D-fraction with specified dosages on tumor-bearing mice or some cancer patients has been documented without defining its physiologic concentrations.<sup>11,14</sup> Therefore, D-fraction may indeed have a potential anticancer activity, and the present study further demonstrates that D-fraction is at least capable of exerting a significant effect on the growth of these canine cancer cells. In addition, the degree of cellular response to D-fraction seems to vary somewhat. For example, both CF33 and CF21 cells were affected moderately and CL-1 cells were affected severely by D-fraction. This finding then indicates that a potency of D-fraction could vary with the cancer cell types.

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### ***D-fraction appears to inhibit the growth of CF33 and CF21 cells and could directly kill CL-1 cells.***

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those cells are literally killed.

I first found that D-fraction was potently effective on three canine cancer cells (CF33, CF22, and CL-1). D-fraction (at 1,060  $\mu\text{g}/\text{ml}$ ) led to the significant (>70%) reduction in numbers of CF33 and CF21 cells but with almost 100% cell viability, while CL-1 cells experienced significant (>90%) death following only a 24-hour D-fraction treatment. Thus, D-fraction appears to inhibit the growth of CF33 and CF21 cells (a growth inhibitory effect) and could directly kill CL-1 cells (a cytotoxic effect). Yet, since the effective concentration of D-fraction appears to be rather high (1,060  $\mu\text{g}/\text{ml}$ ), there is some concern for its use in vivo: It is uncertain whether 1,060  $\mu\text{g}/\text{ml}$  or even 530  $\mu\text{g}/\text{ml}$  of D-fraction could be physiologically achievable.

Because of technical/experimental difficulties, few studies have been conducted to determine the actually attainable physiologic con-

Referring to possible “cancer specificity” of D-fraction, I next examined whether D-fraction might also be effective on human cancer cells. Such studies showed that some human cancer cells responded well to D-fraction. Both HL-60 (leukemia) and T24 (bladder cancer) cells showed significant (>90%) cell death within 24 hours, while the growth of U-87 (brain tumor) cells was profoundly (approximately 90%) inhibited in 3 days. These results suggest that D-fraction is also effective on human cancer cells, demonstrating its growth inhibitory or cytotoxic effects on certain human cancer types. In fact, such a differential efficacy of D-fraction on human cancers has been previously reported.<sup>14</sup> A noncontrolled clinical study of D-fraction on various cancer patients conducted in Japan showed that the clinical status of patients with breast, prostate, lung, and liver cancers was significantly improved

with D-fraction; D-fraction was less effective in patients with bone and gastric cancers.<sup>14</sup> Although this trial is considered an in vivo study, these results seem to reasonably agree with those from my in vitro study. In addition, it is worthwhile to mention that various side effects of chemotherapy on all types of cancer patients were markedly ameliorated when D-fraction was given with conventional drug treatment. This suggests that D-fraction may even greatly improve quality of life in cancer patients receiving chemotherapy.

Lastly, I examined whether other mushroom products on the market might also have a growth inhibitory or cytotoxic effect on canine cancer cells. The results show that three common products (ASC, MSK, and AHC) had no cellular effects on the three cancer cells (CF33, CF21 and CL-1) investigated. Thus, unlike D-fraction, these products appear to be rather ineffective on canine cancer cells tested here. However, the actual efficacy of these products and D-fraction should be assessed using animals (in vivo studies). In the meantime, elaborate toxicity studies of D-fraction were recently performed in animals. A subacute toxicity study using normal, healthy beagles (n = 10) confirmed that no apparent toxicity was observed after D-fraction (3.3 mg/kg/day) was administered for 10 weeks.<sup>a</sup> Another study using mice (n = 20), conducted by a research facility authorized by the Japanese government also demonstrated that a single administration of D-fraction at 2,000 mg/kg (LD<sub>50</sub>) had no toxic/adverse effects on mice.<sup>20</sup> These data may further encourage researchers to conduct D-fraction trials extensively on various animals. Such studies are warranted.

## ■ CONCLUSION

The study discussed here shows that D-fraction is capable of inhibiting cell growth or

<sup>a</sup>Shirota M: Personal communication, Maitake Products, Inc., Ridgefield Park, NJ, 2004.

killing cells in three types of canine cancer cells (CF33, CF21, and CL-1) as well as in certain human cancer cells. However, commercial mushroom products other than D-fraction failed to show such a growth inhibitory or cytotoxic effect on these same canine cancer cells. This study is only preliminary, but the results are highly suggestive that D-fraction could be a promising agent for treating various canine cancers and other veterinary cancers. Thus, further investigations of D-fraction on more animals should be conducted to assess or verify its actual potential as postulated herein.

## ■ ACKNOWLEDGMENTS

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