DOTHISTROMIN RISK ASSESSMENT FOR FORESTRY WORKERS

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ABSTRACT

The fungal toxin dothistromin has the furobenzofuran moiety in common with aflatoxin B1, a known mutagen, human hepatotoxin, and potential human carcinogen. Environmental contamination with dothistromin has been assessed in field studies by sampling forest air, water in the catchment area, and clothing and skin of forestry workers. The possible adverse health effects of such contamination have been assessed retrospectively in two independent epidemiological studies. Dothistromin has also been tested for mutagenicity in a wide variety of *in vitro* bio-assays, most of which have been positive, and for the purpose of a more complete risk assessment, in a mouse *in vivo* mutagenicity assay. Dothistromin appeared to be just as genotoxic as aflatoxin B1 in the mouse bone marrow/peripheral red blood cell assay. The toxin was also detected in the urine of these animals. This work and all the earlier findings with dothistromin have implications for the health and well-being of forestry and other workers.

Keywords: dothistromin; aflatoxin B1; human; hazard; risk; mutagenicity; micronucleus assay; cancer; hepatitis B virus.

INTRODUCTION

The fungus *Dothistroma pini* Hulbary is a needle pathogen of *Pinus* spp. (Gilmour 1967) and produces the mycotoxin dothistromin as a major metabolic by-product (Gallagher & Hodges 1972). Since *D. pini* causes "pine needle blight", a necrosis resulting in premature needle cast, it is of commercial concern in New Zealand in that it affects the health and vigour of commercially grown exotic pine species (Gadgil 1967).

The fungus was first seen in New Zealand in forests of the central North Island in 1964 and is now widespread.

Dothistromin, a difuroanthraquinone, has the same tetrahydro-2-hydroxy-bisfuran moiety as that of aflatoxin B1 from *Aspergillus flavus* (Bassett *et al.* 1970; Gallagher & Hodges 1972; Stoessl & Stothers 1985). It is this particular structural feature of aflatoxin B1 that is considered to be responsible for the hepatotoxicity and potential human carcinogenicity associated with this toxin. *Aspergillus flavus* is a well-known fungal contaminant of stored nuts, grains, and maize, particularly in tropical countries, and has been linked to hepatocellular carcinoma (Van Rensburg *et al.* 1985; Ames *et al.* 1987).

On the strength of the remarkable similarity between dothistromin and aflatoxin B1, mutagenicity studies were initiated with dothistromin. This work for New Zealand has been co-ordinated by the Dothistromin Advisory Working Group (now disbanded) and the New Zealand Department of Health.

In 1973 a mutagenicity assay, using *Neurospora crassa* as the test organism, revealed no mutagenic effect in the presence of dothistromin (R.W. Pero unpubl. data). In 1976, Harvey *et al.* demonstrated that dothistromin inhibited RNA synthesis, as measured by [^{3}H]-uridine incorporation, in *Chlorella pyrenoidosa* and *Bacillus megaterium.* They also observed that dothistromin was 10 times more active than aflatoxin B1 in inhibiting the growth of *B. megaterium.*

In 1984, R.S.U. Baker and other workers (unpubl. data) in a series of three mutagenicity assays (Ames; Sister chromatid exchange [SCE]; Micronucleus [MN]) demonstrated that dothistromin was mutagenic in all three. In the Ames assay, dothistromin was significantly mutagenic in *Salmonella* strains TA97, TA98, and TA100, in the presence of the S9 metabolising system, and as well with strain TA97 in the absence of S9. In the SCE assay, using Chinese Hamster lung cells, dothistromin significantly increased the frequency of SCE's. An exogenous metabolising system was not required for SCE induction. In the MN assay, using the same cell type, dothistromin significantly increased the frequency of MN. An exogenous metabolising system again was not required.

The results of the Ames assay are supported by earlier results obtained by Dr L.R. Ferguson of the Cancer Research Laboratory, Auckland, New Zealand (unpubl. data).

In 1984 Ferguson and other workers extended their work in this area by comparing chromosome damage due to dothistromin in human peripheral blood lymphocyte cultures, with that of aflatoxin B1. Dothistromin caused a dose-dependent increase in the frequency of gaps and deletions which was not dependent on added metabolic activation. Even at high doses of dothistromin only a very small number of complex exchange-type aberrations were seen. This was in contrast with aflatoxin B1, where such aberrations were seen at low dose levels and especially in cultures to which the S9 metabolising system was added. High doses of dothistromin were toxic, manifesting as haemolysis of the donor red blood cells and a reduction in the mitotic index of the white blood cells in the culture system. It was considered that the observed toxicity could be masking any major potential for clastogenicity by dothistromin (Ferguson *et al.* 1986).

In another study, L.R. Ferguson and J.A. McLarin (unpubl. data) examined mutagenesis by dothistromin in V79 Chinese Hamster cells. Dothistromin was a weak mutagen at the 6-thioguanine locus, but only after metabolic activation and there was no effect seen at the ouabain locus. The conclusions from this study were that dothistromin was weakly mutagenic but strongly cytotoxic. It was emphasised that this reflected a narrow difference between effective doses for mutagenicity and toxicity in this compound. The cell toxicity was significantly decreased by metabolic activation.

The cytotoxic propensity of dothistromin was also observed by R.S.U. Baker and others (unpubl. data).

The New Zealand Forest Research Institute supplied the dothistromin, which was isolated from *D. pini,* for all of the above work.

Fungi other than *Dothistroma* are known to produce dothistromin. Dothistromin isolated from *Cercospora arachidicola,* a peanut pathogen, was tested in a brine shrimp assay with the result that 80% of the shrimp were killed with 1 mg/ml within 24 hours. The real significance of this is that dothistromin has now been isolated from a fungal species that is pathogenic to a food crop (Stoessl 1984).

Various field studies have been undertaken, both by the Department of Health in New Zealand and by the Forest Research Institute (FRI). All of this work, and the analysis of the mutagenicity data, relating to dothistromin isolated from *D. pini* was correlated and assessed by the Dothistroma Advisory Working Group in New Zealand during 1984 and 1985.

Extensive environmental monitoring was undertaken in 1984 and 1985 by both the Department of Health and the FRI. Very low levels of dothistromin were found in forest air and in samples from water run-off from the tree canopy, from ponds and streams within the water supply catchment, from *D. pini* spores, and from *D. pini*-infected pine needles. A maximum of 7 ng/ml was found in ponds, streams, and drinking water and less than this in air and run-off from trees (J.K. Briggs unpubl. data; R.A. Franich & P.D. Gadgil unpubl. data).

Human exposure studies undertaken by the Department of Health and the FRI, including examination of dothistromin contamination of worker clothing, skin, and air breathed by forest personnel, showed small but detectable levels of dothistromin. There was greater exposure in wet than in dry conditions, and seasonal and environmental changes were important factors.

Two independent reviews of cancer incidence amongst forestry workers — one by the New Zealand National Health Statistics Centre (NHSC) in Wellington and another by Dr. K. Cooke of the Department of Preventive and Social Medicine of the Otago Medical School, Dunedin, New Zealand — showed no obvious link between cancer of the lung and dothistromin.

Although these studies showed an increased incidence of cancer amongst all wood workers compared to the general population, the conclusion drawn by Dr. Cooke was that this increased incidence was more likely to be due to the irritant effect of dust than to any effect of dothistromin, although dothistromin could not be ruled out entirely as a contributing factor (Anon. 1979–81; Cooke 1983, and unpubl. data).

MATERIALS AND METHODS

Dothistromin

The fungus *D. pini,* supplied by the FRI and subcultured on to Sabouraud's glucose agar, was incubated for 10–14 days at room temperature (20 °C) in the dark. The whole culture in the petri dish, including most of the agar, was then homogenised in a standard laboratory blender to a fine slurry (P.D. Gadgil pers. comm.).The dothistromin from this was then extracted twice with ethyl acetate and the red extract was rotary evaporated to dryness and purified essentially according to the method of Shain & Franich (1981). We used agar-based media for dothistromin isolation because, in our studies and studies undertaken at the FRI, greatly increased yields of

dothistromin were obtained on solid media compared to broth. Others have also noted this (Stoessl & Stothers 1985).

Mouse Micronucleus (MN) Assay

The mouse bone marrow/peripheral red blood cell (erythrocyte) assay was performed essentially according to Schlegel & MacGregor (1982). Four groups of five male mice, selected at random and weighing approximately 40 g, were injected intraperitonealy with (1) vehicle, consisting of dimethylsulphoxide in saline, (2) 0.1 mg dothistromin/kg, (3) 1.0 mg dothistromin/kg, and (4) 1.0 mg aflatoxin B1/kg on Day 1 and again on Day 4. The mice were weighed before treatment and just prior to sacrifice. Immediately after cervical dislocation, cardiac puncture was performed and blood smears were prepared. The two femora were removed and the marrow was flushed into a test tube containing calf serum, the tubes were centrifuged and smears prepared. All smears were stained by the May-Grunwald-Giemsa method (Matter & Schmid 1971) Forty thousand normochromic erythrocytes (NCE) were examined per slide in the peripheral blood smear preparations and the percentage of MN was recorded. Polychromatic erythrocytes (PCE) were also noted and the NCE/PCE ratio was examined (Table 1). The marrow smears were also examined but micronuclei were not scored in these.

Urine was collected from the group of mice injected with vehicle alone and also from the group injected with the highest dose of dothistromin.

TABLE 1—Mouse micronucleus assay

Drug treatment	Dose (mg/kg)	Weight (g) B	Weight (g) A	MN/1000NCE	NCE/PCE
Vehicle	0	42.4	41.5	0.73 ± 0.13	n
Dothistromin	0.1	38.1	38.3	0.88 ± 0.10	n
Dothistromin	1.0	39.4	39.9	1.00 ± 0.25	n
Aflatoxin	1.0	39.1	40.5	0.91 ± 0.36	n

Dose — agent given twice i.p. (*see* text)
Vehicle is DMSO/saline.
Weight in grams B = before treatment, A = end of treatment
MN/1000NCE ± standard deviation
NCE/PCE, where n = normal ratio indicating nil toxicity

RESULTS AND DISCUSSION

Apart from the first mutagenicity test conducted in 1973, all of the subsequent tests have shown that dothistromin is a mutagen.

Our work appears to be the first *in vivo* mutagenicity study attempted with dothistromin. Micronuclei were scored in peripheral blood NCE's as recommended by Schlegel & McGregor (1982) and Choy *et al.* (1985). They have shown that micronucleated erythrocytes have a normal lifespan in the mouse peripheral circulation and that the frequency of MN in circulating NCE's reflects the clastogenic effect observed in the cells in the bone marrow.

Here we have compared dothistromin with aflatoxin B1 in order to determine individual and comparative activity. Both aflatoxin and dothistromin caused an increase in the number of micronucleated NCE's in the peripheral circulation of the mice over that of the vehicle alone (Table 1).

Micronuclei result either from damage to the spindle apparatus or as a result of chromosome damage (clastogenicity) in the dividing erythroblast and reflect generally what would happen to any somatic cell, and possibly germinal cells in the body. Both of these events can lead to whole chromosomes or chromosome fragments not being incorporated into daughter nuclei at mitosis and forming MN. There is a recognised low spontaneous frequency of these.

The increased numbers of MN seen were not statistically significant (Student's *t* test). This was thought to be due in part to the use of random rather than inbred mice which resulted in relatively high individual scatter in MN levels. The assay showed, however, that dothistromin and aflatoxin at the same dose appeared to be of equivalent clastogenic potency.

The number of polychromatic (young) erythrocytes (PCE's) to NCE's remained constant, indicating no toxic effect on bone marrow erythroblasts, and the weight of the animals also remained constant over the week (Table 1).

Urine collected from the group of mice injected with the highest dose of dothistromin (one dose), when extracted with ethyl acetate and assayed by TLC, revealed dothistromin and other metabolites. This finding is of some concern because it shows that dothistromin at 1 mg/kg was not completely detoxified in the animals.

A Canadian report (Anon. 1986) promoted criteria relating to the potential hazard of mutagenic chemicals in the environment. The report established what were termed "levels of concern" for chemicals. If chemicals are shown to be negative in mutagenicity tests, the concern is zero. At the other end of the scale where both *in vitro* and *in vivo* mutagenicity assays are positive, the concern is such that restricted exposure to the chemical is recommended. Where a chemical has been shown to be linked to human cancer, such as aflatoxin B1 (Van Rensburg *et al.* 1985), then the recommendation is that exposure be eliminated. This, of course, is the ideal and not necessarily what can be achieved in practice. The Canadian document addresses the topic of hazard assessment but not of risk.

There is now sufficient evidence to show that dothistromin can be considered a potential carcinogen; it may not act quite like aflatoxin or be as potent a mutagen, but a mutagen nevertheless it is.

Weak mutagens can be potent carcinogens and potent mutagens, non-carcinogens. For example, sodium azide used as a anti-bacterial agent is a powerful mutagen but is non-carcinogenic. Some agents are, in turn, carcinogenic but not mutagenic (Ames *et al.* 1987). Some of the aflatoxins (for example, B2a and G2a) are mutagenic but have not been shown to cause cancer (Gallagher 1971).

Dothistromin can be labelled a potential human hazard, but is it a risk? We have used the "weight of evidence" approach to assess the possible human health risk associated with dothistromin. The risk factors are summarised in Table 2 and the "anti"-risk and risk-reducing factors in Table 3.

TABLE 2—Dothistromin risk factors

Mutagenic
Clastogenic
Cytotoxic
Worker contamination established
Wet conditions more hazardous
High levels of infection in some forests
Degrades slowly in decaying needles
Weak mutagens can be potent carcinogens
Ames prediction (Maron & Ames 1983)
(80% of animal carcinogens are also bacterial mutagens)

TABLE 3—Dothistromin "anti"-risk factors and risk-reducing factors

1. Dothistromin "anti"-risk factors
No compelling epidemiological evidence of increased cancer association
Behaves differently to "model" — aflatoxin
Weak mutagen (not always a good guide)
Not all mutagens are carcinogens
Some aflatoxins are not carcinogens
Miniscule amounts in environment
Cytotoxicity may be the major toxic effect (this in itself may lead to genotoxicity)
Metabolism reduces toxicity but does not assist mutagenicity as with aflatoxin
DNA repair enzymes are present in cells
Workers can be easily protected and educated to the hazard
2. Dothistromin risk-reducing factors
Control forest infection to <15%
Worker protection —
• cotton clothes rather than wool
• wash clothes regularly
• good personal hygiene
• wear face masks
Forest managers should schedule work in dry rather than wet conditions

When comparing aflatoxin with dothistromin, it can be seen that *in vitro* metabolic activation increases aflatoxin mutagenicity, whereas it does not appreciably alter dothistromin mutagenicity, although abolishing its toxicity. Thus detoxification allows dothistromin to act principally from that point on as a mutagen.

The conclusion from these findings is that accumulation of dothistromin in tissues with high metabolic activity (for example, the liver) may lead to mutagenic and possibly carcinogenic effects, while accumulation in tissues with low metabolic activity (for example, skin and lung) may lead to toxicity without mutagenic, and the possibility of carcinogenic, consequences.

There are, however, riders to these conclusions. Toxicity is not always innocuous, and the release of mutagenic oxygen radicals from poisoned cells and host phagocytic

cells can have an effect on surrounding cells (Ames *et al.* 1987). The nucleated cells of the body contain DNA repair enzymes which repair all damage most of the time. Skin cells and lung cells, for example, are shed regularly and mucous membranes are "protected" in the gut and nasopharynx by the mucous they secrete.

Epidemiological studies have not shown that dothistromin can be linked to lung cancer. Liver cancer was not specifically addressed in these reports, but presumably wood workers in general do not suffer from an increased incidence of this disease.

When trying to put the risk from dothistromin into perspective, we believe it is legitimate to compare it with aflatoxin. Animal cancer studies with aflatoxin have shown that 50 ng/kg body weight/day was the minimum dose needed to produce cancer (Dothistroma Advisory Working Group, unpubl. data). Dothistromin levels as determined in the environmental and human exposure monitoring studies were considerably lower than this. Ames *et al.* (1987) have estimated that one peanut butter sandwich (in America) contains about 64 ng of aflatoxin. They also claim that cancer death rates in America, except for lung cancer due to tobacco and melanoma due to UV light, are not on the whole increasing and, indeed, have mostly been steady for the past 50 years. This suggests that possibly hygiene, combined with natural defences, is limiting the potential effect of environmental carcinogens.

Dothistromin and aflatoxin are, in fact, only two of about 300 known mycotoxins and most of these are mutagens and animal carcinogens (Ames *et al.* 1987). Mycotoxins in general are seen as common pollutants of human food, particularly in the tropics.

Van Rensburg *et al.* in a 1985 study in Mozambique and the Transkei on hepatocellular carcinoma and its association with dietary aflatoxin, concluded that there is very strong evidence for a link between liver cancer, aflatoxin, and the hepatitis B virus (HBV). As a comparison, in areas where there is a high prevalence of HBV and a low incidence of liver cancer, such as in Greenland, then this parallels the low level of environmental aflatoxin, being too cold for the growth of *Aspergillus flavus*. They suggested that HBV is the initiator and aflatoxin the promoter, which together, but not either alone, can lead to liver cancer.

In conclusion then, it may well be that although acting in slightly different ways, dothistromin might be just as hazardous as aflatoxin and that neither of these environmental toxins is much of a human health risk, unless there is some underlying pathology. This predisposing debilitation could, for example, be due to the effects of smoking or to HBV infection.

We have no wish, however, to down play the continuing need for proper hygiene and the wearing of adequate protective clothing for any worker who has contact with dothistromin. It is known that dothistromin can be completely destroyed by dissolution in alkaline solution (e.g., soap) and exposure of the solution to air and light. Conditions leading to oxidative degradation of dothistromin have been summarised by Franich *et al.* (1986).

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REFERENCES

AMES, B.N.; MAGAW, R.; GOLD, L.S. 1987: Ranking possible carcinogenic hazards. *Science 236:* 271–9.

ANON. 1979–1981:"A Review of Cancer Incidence Among Forestry Workers Aged 15–64". National Health Statistics Centre, Wellington, New Zealand.

ANON. 1986: Guidelines on the use of mutagenicity tests in the toxicological evaluation of chemicals. A report of the DNH & W/DOE Environmental Contaminants Advisory Committee on Mutagenesis, Ottawa, Canada.

BASSETT, C.; BUCHANAN, M.; GALLAGHER, R.T.; HODGES, R. 1970: A toxic difuroanthraquinone from *Dothistroma pini. Chemistry and Industry 52:* 1659–60.

CHOY, W.N.; MacGREGOR, J.T.; SHELBY, M.D.; MARONPOT, R.R. 1985: Induction of micronuclei by benzine in B6C3F1 mice: Retrospective analysis of peripheral blood smears from the NTP carcinogenesis bioassay. *Mutation Research 143:* 55–9.

COOKE, K.R. 1983: Lung cancer in different occupational unit groups. DIH Thesis, Otago University Medical School.

FERGUSON, L.R.; PARSLOW, M.I.; McLARIN, J.A. 1986: Chromosome damage by dothistromin in human peripheral blood lymphocyte cultures: a comparison with aflatoxin B1. *Mutation Research 170:* 47–53.

FRANICH, R.A.; CARSON, S.D.; CARSON, M.J. 1986: Synthesis and accumulation of benzoic acid in *Pinus radiata* needles in response to tissue injury by dothistromin, and correlation with resistance of *P. radiata* families to *Dothistroma pini. Physiological Plant Pathology 28:* 267–86.

GADGIL, P.D. 1967: Infection of *Pinus radiata* needles by *Dothistroma pini. New Zealand Journal of Botany 5:* 498–503.

GALLAGHER, R.T. 1971: The structure of dothistromin. Ph.D. Thesis, Massey University, Palmerston North.

GALLAGHER, R.T.; HODGES, R. 1972: The chemistry of dothistromin, a difuroanthraquinone from *Dothistroma pini. Australian Journal of Chemistry 25:* 2399–407.

GILMOUR, J.W. 1967: Host list for *Dothistroma pini* in New Zealand. *Forest Research Institute, Research Leaflet No. 16.*

HARVEY, A.M.; BATT, R.D.; PRITCHARD, G.G. 1976: Inhibition of RNA synthesis in *Chlorella pyrenoidosa* and *Bacillus megaterium* by the pine-blight toxin, dothistromin. *Journal of General Microbiology 95:* 268–76.

MARON, D.M.; AMES, B.N. 1983: Revised methods for the Salmonella mutagenicity test. *Mutation Research 113:* 173–215.

MATTER, B.; SCHMID, W. 1971: Trenimon-induced chromosomal damage in bone marrow cells of six mammalian species, evaluated by the micronucleus test. *Mutation Research 12:* 417–25.

SCHLEGEL, R.; MacGREGOR, J.T. 1982: The persistence of micronuclei in peripheral blood erythrocytes: detection of chronic chromosome breakage in mice. *Mutation Research 104:* 367–9.

SHAIN, L.; FRANICH, R.A. 1981: Induction of Dothistroma blight symptons with dothistromin. *Physiological Plant Pathology 19:* 49–55.

STOESSL, A. 1984: Dothistromin as a metabolite of *Cercospora arachidicola. Mycopathologia 86:* 165–8.

STOESSL, A.; STOTHERS, J.B. 1985: Minor anthraquinonoid metabolites of *Cercospora arachidicola. Canadian Journal of Chemistry 63:* 1258–62,

VAN RENSBURG, S.J.; COOK-MOZAFFARI, P.; VAN SCHALKWYK, D.J.; VAN DER WATT, J.J.; VINCENT, T.J.; PURCHASE, I.F. 1985: Hepatocellular carcinoma and dietary aflatoxin in Mozambique and Transkei. *British Journal of Cancer 51:* 713–26.