

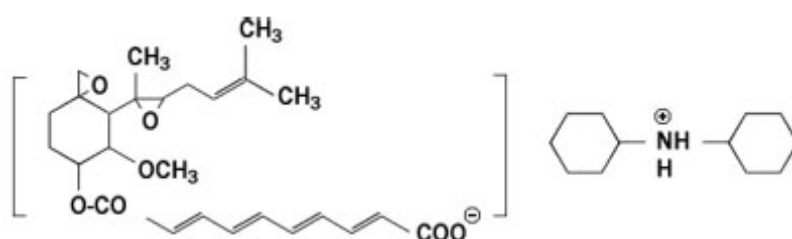
COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

UPDATED STATEMENT ON THE EVALUATION OF GENOTOXICITY DATA OF FUMAGILLIN DICYCLOHEXYLAMINE (2011)

Introduction

1. Fumagillin dicyclohexylamine (fumagillin DCHA) is an antibiotic authorised for use in honey bees for the prevention of infections caused by the *Nosema apis* parasite present in the gut of infected bees (Figure 1). Fumagillin DCHA is fed to the colony in winter over a period of several weeks in a medicated syrup as a supplementary food source to eradicate the parasites. The commercial formulation of fumagillin DCHA is a stabilised water-soluble preparation, Fumidil-B (CEVA Animal Health). Fumidil-B contains the excipient polysorbate 80, sodium phosphate (anhydrous) and sodium acid phosphate (anhydrous). The veterinary medicine Marketing Authorisation Holder (MAH) is CEVA Animal Health.

Figure 1. Structure of Fumagillin DCHA Salt



Background

2. In February 2009, the COM considered studies investigating *in-vitro* chromosomal aberrations, primary DNA damage unscheduled DNA synthesis (UDS), *in-vivo* micronucleus and primary DNA damage studies (UDS and Comet), undertaken by Nesslany, and a critical analysis of mutagenicity data of fumagillin DCHA salt, all submitted by the MAH. In addition, the MAH submitted data on thermal and degradation studies with fumagillin DCHA, stability and genotoxicity studies with dicyclohexylamine, a summary of the genotoxicity data with fumagillin DCHA, a critical analysis of the Nesslany studies and those published by the Stanimirovic group, additional genotoxicity data on fumagillin acid and information on the mode of fungicidal action of

fumagillin. A tabulation of the key studies and results is appended as table 1 in Annex 1¹⁻¹⁰. Positive results had been reported for *in vitro* clastogenicity by both the Nesslany^{1,2} and Stanimirovic groups³⁻⁵. However evidence for an *in vivo* clastogenic effect had been reported in the Stanimirovic studies^{6,8} but not in those undertaken for the MAH by Nesslany.^{7,9,10}

3. The COM Secretariat retrieved additional published data on the stability of fumagillin and fumagillin DCHA, and genotoxicity studies on fumagillin DCHA and dicyclohexylamine.¹¹⁻²¹ This included the papers published by the Stanimirovic group on *in-vitro* studies investigating chromosomal aberrations, sister chromatid exchanges, *in-vivo* chromosome aberration and micronucleus studies with fumagillin DCHA. The MAH also made a presentation to the Committee at its February 2009 meeting and responded to questions.^{22,23}

4. The COM agreed that the Stanimirovic data were limited and no definite conclusions could be reached. There were several possible explanations for the differences between the results obtained for *in-vivo* genotoxicity studies undertaken by the Stanimirovic group and the MAH. These included possible differences in absorption, metabolism of the administered test material, differences in stability of the test materials including storage, and different impurity profiles between test materials used by the research groups. The COM agreed that the genotoxicity data on fumagillin acid and dicyclohexylamine tested separately did not provide sufficient information to draw conclusions on the role of these substances in the potential mutagenicity of fumagillin DCHA.

5. The COM recommended the following testing strategy for fumagillin DCHA

i). A further *in-vivo* mutagenicity study using the same protocol used by Stanimirovic et al. (*Mutat Res* (2007) 628, 1-10)³ to include sampling of bone marrow for MN and chromosomal aberrations.

ii) A site of contact comet assay using gastrointestinal (stomach) tissue. (The comet assay should also include an appropriate positive control substance).

iii). If any genotoxicity is observed with fumagillin DCHA, more genotoxicity data (*in-vitro* chromosomal aberration test in human lymphocytes) should be provided on dicyclohexylamine to evaluate its potential role. (Any study should also include fumagillin DCHA for quantitative comparison).

Referral

6. The VMD asked the COM the following:

a) To ask the COM for advice on the interpretation of the additional studies

b) To ask the COM for its final opinion (following the additional studies) on the potential for fumagillin dicyclohexylamine as a mutagen and an *in vivo* clastogen

Data Submitted to COM

7. The same batch of Fumagillin DCH was used for all tests; 9002385/B containing 61.75% Fumagillin acid, anhydrous. Test material was stored under cool conditions (ca 2-8°C) in the dark. Test material was administered in a 1:1 sugar/water syrup.

Chromosome Aberration Study in the Mouse (Covance)²⁴

8. Fumagillin DCHA was tested for its ability to induce chromosome aberrations in the bone marrow of male Crl:CD-1(ICR) mice when administered orally by gavage at 25, 50 and 75 mg/kg bw/day in a dose volume of 10ml/kg water-sugar syrup solution over 7 days. The positive control was CPA administered at 40 mg/kg bw in a single dose 16 hours prior to the bone marrow harvest. The bone marrow was sampled 16 hours post the final administration.

9. Blood samples were taken at 2h post dose from satellite animals treated with a single dose of 75 mg/kg bw fumagillin DCHA. Plasma concentrations of fumagillin DCHA were determined.

Bone Marrow Micronucleus Test with Comet Assay in Stomach of Mouse (Institut Pasteur)²⁵

10. Fumagillin DCHA (suspended in a sugar/water 1:1 syrup) was administered orally to groups of 5-7 male and female OF1 mice on 8 consecutive days at dose levels of 25 mg/kg bw/day, 50 mg/kg bw/day and 75 mg/kg bw/day in the main study. The animals were killed 3-6 hours after the final dose and bone marrow isolated for micronucleus determination in polychromatic erythrocytes (male and females) and stomach for comet assay (males). Positive controls were cyclophosphamide (50 mg/kg bw, i.p 5 male/5 female mice) for BMMN and MNNG (20 mg/kg bw p.o in 4 male mice) for the comet assay. A complementary (additional) comet assay (stomach) study was undertaken using dose levels of 5, 10, 25 mg/kg bw/day.

11. Blood samples were taken at 2h post dose from satellite animals treated with a single dose of 25, 50 or 75 mg/kg bw fumagillin DCHA. Plasma concentrations of fumagillin DCHA were determined

COM Discussion

General aspects

13. The Committee noted there had been considerable problems in the preparation of dosing solutions of Fumagillin in sugar/water syrup. In addition there had been significant treatment-related toxicity and mortality, particularly in female mice which complicated the interpretation of these studies. Members noted that Fumagillin DCHA was virtually insoluble in water. Achieved concentrations were almost always lower than nominal, there was difficulty in attaining homogeneous solutions and the possibility of degradation could not be excluded. Nominal doses are referred to in the text. However the kinetic data had shown comparable systemic exposure in the studies undertaken by Covance and the Institut Pasteur. The Committee agreed that the submitted studies had adequately addressed the data requests agreed by COM in 2009.

Chromosome Aberration Study in the Mouse (Covance)

14. The COM agreed this study had been adequately conducted and had replicated the study undertaken by the Stanimirovic group and had reported negative results.

Bone Marrow Micronucleus Test with Comet Assay in Stomach of Mouse (Institut Pasteur)

15. The Committee agreed there had been systemic uptake and treatment related toxicity in male (decreased body weight gain) and in female animals (reduced body weight gain and mortality at 50 or 75 mg/kg bw/day). An increase in comet formation (Olive Tail Moment) was found at 25 mg/kg bw/day in the main study which was accompanied by an increase in ghost cells indicative of a cytotoxic response. The Committee agreed there were a number of factors which limited the weight of evidence that could be attached to results reported from the Comet assay. These limitations included the observation that negative and positive control comet data overlapped indicating limitations in the performance of the comet assay at this laboratory. In addition the investigations of trypan blue exclusion for necrosis was limited since stomach tissue had been treated with trypsin. The Committee also noted that the Halo assay was of limited use in assessing necrosis and apoptosis.

16. A complementary (additional) Comet assay (stomach) study was undertaken in males using dose levels of 5, 10, 25 mg/kg bw/day for eight days. Treatment-related mortality had been reported at all dose levels. In the complementary assay, an apparent dose-related decrease in comet formation was observed. The Committee agreed that in this instance, in view of the extent of toxicity reported, that no conclusions could be reached with regard to the significance of this observation.

17. The COM agreed the bone marrow micronucleus tests had been adequately conducted and had replicated the study undertaken by the Stanimirovic group and had reported negative results.

COM Conclusions

18. The newly submitted bone marrow chromosome aberrations and micronucleus tests in mice adequately addressed the data requests agreed by COM in 2009, and reported negative results. There were limitations in the conduct of the *in vivo* Comet assay in mice; overall a negative result was reported.

19. Fumagillin DCHA should be considered as an *in vitro* mutagen.

20. The weight of evidence available to the COM supports the conclusion that Fumagillin DCHA is not an *in vivo* mutagen.

July 2011

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Table 1. Summary of Fumagillin DCHA Genotoxicity Data (Annex 1)

Study	Reference	Date	Test system	Test material	GLP	Dose/concentration range	Result
1. In-Vitro Studies							
1.1 Bacterial gene mutation	Jones <i>et al.</i> (Cited in CVMP opinion)	1985	TA1535, 1537, 1538, 98 & 100 +/- S9 two independent assays	Fumagillin DCHA (60 %) in water	Yes	50-5000 µg/plate	Negative
	Bela (Cited in CVMP opinion)	1989	TA1535, 1537, 97a, 98, 100 & 102 +/- S9 2 independent assays	Fumagillin DCHA (? %) in DMSO	Yes	78.1-5000 µg/plate	Negative
	Nesslany	2004	TA1535, 1537, 98, 100 & 102 +/- S9 2 independent assays	Fumagillin DCHA	Yes	50-3000 µg/plate	Negative
1.2 Chromosomal effects							
1.2.1 Chromosome aberration studies	Allen (Cited in CVMP opinion)	1985	CHO 2 hours + S9, 20 hours – S9	Fumagillin DCHA (60 % ?) in water	Yes	10-100 µg/ml	Negative * No repeat assay Out of spec material
	Nesslany^{1,2}	2006	Human lymphocytes 4 hours +/- S9 5% 4 hours + S9 10% 20 hours –S9 44 hours –S9	Fumagillin DCHA (62.86%) in 0.5% ethanol	Yes	177.78-400 µg/ml 118.52-266.67 µg/ml 35.56 -80 µg/ml 35.56- 80 µg/ml	Positive ≥ 266.7 µg/ml Positive ≥ 177.78 µg/ml Positive ≥ 53.33 µg/ml Positive ≥ 35.36 µg/ml
	Nesslany²	2007	Human lymphocytes 44 hours –S9	Fumagillin DCHA as above	Yes	4.39 – 33.33 µg/ml	Positive ≥ 6.58 µg/ml
	Stanimirovic <i>et al.</i>^{3,4} Stevanovic <i>et al.</i>⁵	2007 2008	Human lymphocytes 24 hours? –S9	Fumagillin DCHA in sugar-water	NS	1.02 – 9.20 µg/ml 0.34 – 9.20 µg/ml	Positive ≥ 3.07 µg/ml
1.2.2 Sister chromatid exchange	Stanimirovic <i>et al.</i>^{3,3} Stevanovic <i>et al.</i>⁵	2007 2008	Human lymphocytes 24 hours? –S9	Fumagillin DCHA in sugar-water	NS	1.02 – 9.20 µg/ml 0.34 – 9.20 µg/ml	Positive ≥ 1.02 µg/ml
1.3 Primary DNA damage Unscheduled DNA synthesis	Bichet (Cited in CVMP opinion)	1993	Rat hepatocytes autoradiography method	Fumagillin DCHA (52.3 – 54.2%) Fumagillin 'Impurities' DCH in ethanol	Yes	50 – 500 µg/ml 75, 100 µg/ml 100 – 250 µg/ml 250 µg/ml	Equivocal

Table 1. cont'd Summary of Fumagillin DCHA Mutagenicity Data

Study	Reference	Date	Test system	Test material	GLP	Dose/concentration range	Result
2. In-Vivo Studies							
2.1 Chromosomal effects							
2.1.1 Chromosome aberration studies	Molinier ¹³	1992	Chinese hamster bone marrow sampled 6, 24 & 48 hours after dosing	Fumagillin DCHA (61.59%) in ?	Yes	Single i.p. dose level NS	Negative * Out of spec material Single dose level Inadequate scoring
	Stanimirovic et al. ⁶	2007	Male BALB/c mouse bone marrow	Fumagillin DCHA in sugar-water solution	NS	25, 50, 75 mg/kg p.o. daily for 7 days	Positive at all doses
2.1.2 Micronucleus studies	Kiss (Cited in CVMP opinion)	1989	Male/female NMRI mouse bone marrow	Fumagillin DCHA (62%) in 0.9% NaCl	Yes	Single i.p. dose 150 or 300 mg/kg sampled at 30, 48 or 72 hours or two i.p. doses 24 hours apart sampled at 24 & 48 hours	Equivocal Small increase in males at 300 mg/kg dose. Small increase in females with dose response. Signif. increase in both sexes after 2 doses
	Nesslany ⁷	2006	Male/female SD rat bone marrow sampled 24 hours after treatment	Fumagillin DCHA (62.86%) in 0.5% CMC suspension	Yes	2 p.o. doses 24 hrs apart 160, 320, 500 mg/kg males, 80, 160, 320 females	Negative
	Stevanovic et al. ⁸	2007	Male/female BALB/c mouse bone marrow	Fumagillin DCHA in sugar-water solution	NS	25, 50, 75 mg/kg p.o. daily for 7 days	Positive at all doses
2.2 Primary DNA damage							
2.2.1 Unscheduled DNA synthesis	Nesslany ⁹	2004	Male Fischer rat hepatocytes 2-4 & 12-16 hour expression times	Fumagillin DCHA (72.8%) in 0.5% CMC suspension	Yes	125, 250 mg/kg p.o. single dose	Negative
2.2.2 Comet assay	Nesslany ¹⁰	2006	Male SD rat hepatocytes, 3-6 & 22-26 hour expression times	Fumagillin DCHA (62.86%) in 0.5% CMC suspension	Yes	400 , 800 mg/kg p.o. single dose	Negative

* These studies are not considered valid (Cited in CVMP opinion) and summarised in MUT/2009/01, in confidence paper.

