Acta Veterinaria (Beograd), Vol. 56, No. 5-6, 437-448, 2006.

DOI: 10.2298/AVB0606437S UDK 619:615.06

MONITORING OF MITOTIC INDEX AND FREQUENCY OF MICRONUCLEI IN EVALUATION OF GENOTOXIC POTENTIAL OF FUMAGILLIN (DICYCLOHEXYLAMINE) *IN VIVO*

STEVANOVIĆ JEVROSIMA*, STANIMIROVIĆ Z*, PEJIN I IVANA* and LAZAREVIĆ M**

*Department of Biology, Faculty of Veterinary Medicine, Belgrade
**Department of Physiology and Biochemisty, Faculty of Veterinary Medicine, Belgrade

(Received 2. February 2006)

Fumagillin (dicyclohexylamine) is a natural antibiotic, secreted by Aspergillus fumigatus. It is used in veterinary medicine against microsporidiosis in bees and fish, as well as in human medicine for the treatment of intestinal amebiasis, microsporidial keratoconjunctivitis and intestinal microsporidiosis due to Enterocytozoon bieneusi in patients with AIDS and other types of immunodeficiency. In this study, the genotoxicity of fumagillin was evaluated in mouse bone marrow cells using the mitotic index (MI) and micronucleus (MN) assay. Fumagillin was administered to BALB/c mice by gavage in doses of 25, 50, 75 mg/kg b.w., repeated for 7 days at 24h intervals, with water-sugar syrup as the negative control and cyclophosphamide as the positive control (40 mg/kg b.w.) All experimental doses of fumagillin induced a significant decrease ($p<0.001$) in MI (3.47 ± 0.04%, 3.17 ± 0.01% and 2.27 ± 0.02%, respectively) in comparison with the negative control (6.00 ± 0.01%) and with the positive control (14.78 ± 0.09). Fumagillin significantly ($p<0.001$) increased the frequency of MN (4.98 ± 0.35, 8.45 ± 0.57 and 12.02 ± 0.37, respectively) over the negative control (1.04 ± 0.28). These results suggest that fumagillin (dicyclohexilamine) has an antiproliferative and genotoxic potential in mammal in vivo test.

Key words: Fumagillin (dicyclohexylamine), Honey bee, Nosema-disease, Genotoxicity, Mitotic index (MI), Micronuclei (MN)

INTRODUCTION

Fumagillin, an antibiotic derived from the fungus *Aspergillus fumigatus*, has particular activity against microsporidia and various amoeba species (McCowen *et al*., 1951; Killough *et al*., 1952; Katznelson and Jamieson, 1952; Bailey, 1953; Shadduck, 1980; Didier, 2005). It was first reported to be active against *Nosema apis* in honey bees (*Apis mellifera* L.) in the early 1950's (Katznelson and Jamieson, 1952; Bailey, 1953).

Fumagillin is quite stable in honey bee hives (Furgala, 1962). Furthermore, investigations of Assil and Sporns (1991) showed that fumagillin was very stable in honey at elevated temperatures (stable for at least 35 days at 80^{o}C). The use of

fumagillin is permitted in the EU and the USA (EMEA, 2000; FDA, 2005), but the maximum residue level (MRL) is not affirmed neither in the EU, nor in the USA.

Fumagillin has also been proposed for suppression of other microsporidian parasites in both invertebrates and vertebrates. Thus, it is revealed that fumagillin is effective against *Octosporea bayeri* in the freshwater crustacean *Daphnia magna* (Zbinden *et al*., 2005) and *Myxobolus cerebralis* (Myxosporea), which is the causative agent of whirling disease in many fish species (El-Matbouli and Hoffmann, 1991; Karagouni *et al*., 2005) and *Tetracapsuloides bryosalmonae* (Malacosporea), which is the causative agent of proliferative kidney disease of chinook salmon *Oncorhynchus tshawytscha* (Hedrick *et al*., 1988; Kent and Dawe, 1994) as well as rainbow trout *Oncorhynchus mykiss* (Le Gouvello *et al*., 1999; Morris *et al*., 2003). However, in more rigorous tests required for the U.S. Food and Drug Administration approval, it was found to be ineffective (Gilbert and Granath, 2003).

In humans, fumagillin is used for the treatment of intestinal amebiasis (McCowen *et al*., 1951; Killough *et al*., 1952). It is effective when used topically in the treatment of microsporidial keratoconjunctivitis (Roseger *et al*., 1993; Wilkins *et al*., 1994) and orally in the treatment of intestinal microsporidiosis due to *Enterocytozoon bieneusi* in patients with AIDS and other types of immunodeficiency (Molina *et al*., 2000, 2002; Conteas *et al*., 2000).

It was revealed that fumagillin has the ability to inhibit endothelial proliferation and block angiogenesis *in vitro and in vivo*. However, administration of fumagillin is limited because of its toxic side-effects (Ingber *et al*., 1990, Didier, 2005). Analogues with fewer side-effects have been synthesized, and one of these, TNP-470 (AGM-1470), was shown to inhibit tumor growth in a number of xenograft models (Yamaoka *et al*., 1993; Yanase *et al*., 1993; Kurebayashi *et al*., 1994; Tanaka *et al*., 1995). However, TNP-470 was found to possess immunosuppressive activity (Turk *et al*., 1998) and produces excessive cytotoxicity (Ingber *et al*., 1990; Ruddy *et al*., 2000). Moreover, in studies of Yoshida *et al*. (1998) TNP-470 inhibited the production of leukemia inhibitory factor, which may be related to the amelioration of cancer cachexia. TNP470 induced apoptosis and enhanced the expression of P-galactosidase, a biomarker of senescence (Yoshida *et al*., 1998).

Recent investigations of Huang *et al*. (2004) have indicated that the antitumor effects of TNP-470 cannot be attributed to the prevention of neoangiogenesis, but instead to its direct action on tumor cells.

Data refering to genotoxic effects of fumagillin were obtained by *in vitro* investigations and the results were either positive (Stoltz *et al*., 1970; Stanimirovic *et al*., 1999; Stevanovic *et al*., 2000) or negative (Purchase *et al*., 1978; Mortelmans *et al*., 1986; Heil *et al*., 1996). In addition, there are no references regarding genotoxic effects of fumagillin *in vivo* (Toxicological Evaluation, 2000).

All listed information point to a necessity of investigations of genotoxic effects of fumagillin *in vivo*. The objective of this study was to evaluate and characterize by the micronucleus assay the possible genotoxic effects of fumagillin *in vivo*, by using different doses which are in the therapeutic range in

beekeeping. The endpoints used for genotoxic analysis were micronuclei (MN) and mitotic index (MI) in mouse bone-marrow cells.

MATERIAL AND METHODS

The genotoxic effects of fumagillin (Fumagillin-ET, 3/0-05-009/001- JKL 022; Evrotom, Ruma, SCG) were investigated in bone marrow cells of BALB/c strain mice, using the micronucleus (MN) test system. The mitotic index (MI) was also determined to detect the cytotoxic effect of fumagillin.

Three experimental doses of fumagillin were tested: 25, 50 and 75 mg/kg b.w. The median experimental dose of 50 mg/kg corresponds to the therapeutic dose-range of fumagillin in beekeeping, since the recommended fumagillin dose for honey bees is 26 mg fumagillin/L (Webster, 1994).

Fumagillin does not dissolve readily in water. To prepare the medicated sugar syrup, it is recommended to mix fumagillin in small amounts of warm water (not above 32-34^{o}C) and stirit into a paste, then add the prepared water-sugar syrup gradually and shake the container occasionally. The antibiotic mixture should be admixed with water-sugar syrup shortly before use.

The experimental design for micronucleus (MN) assay included three groups: positive control, negative control, and the treated group. The treated groups were divided into three subgroups based on selected doses of fumagillin. All groups had eight animals per dose/group (both sexes). Our experimental design was carried out in two cycles. We used BALB/c mice of 6 weeks of age with an average weight of 19±2 g. Animals were kept under uniform conditions and were housed under 12/12-h photoperiod at constant temperature (21^{o}C) with free access to standard laboratory chow and water.

Experimental doses were obtained by dissolving fumagillin in 1:1 water-sugar syrup orally administered to mice, as is the case with the formulation usually used in beekeeping.

The negative control group was treated *per os* with water-sugar syrup. A known mutagen, cyclophosphamide at a dose of 40 mg/kg b.w. was used as the positive control group due to its known clastogenic and mutagenic features (Anderson *et al*., 1995). Cyclophosphamide was intraperitoneally (i.p.) administered, and the injected volume was 0.01 mL/g b.w. All animals received daily i.p. treatments for a 7-day duration.

Schmid method (Schmid *et al*., 1975) was used for the analysis of micronuclei (MN) in polychromatic erythrocytes (PCE) of mouse bone marrow. At least four slides were made for each animal and allowed to dry overnight and later stained with May–Gruenwald–Giemsa (MOL d.d., Beograd, SCG) solutions according to the standard technique (Adler, 1984) for conventional assessment of MN frequencies. All slides were coded for microscopic analysis at 1000x magnification. Per animal, 1000 polychromatic erythrocytes (PCE) from each of four slides were scored for the presence of MN. The mitotic index (MI) was determined on 1000 or more cells in accordance with the guidelines of Zimonjic *et al* (1990).

Statistical analyses were carried out with the software programme Statistica 6.0 using the Analysis of Variance (ANOVA) and the Student's *t*-test and LSD-test.

RESULTS

The effects of fumagillin were evaluated in bone marrow cells of BALB/c strain mice by monitoring the mitotic index (MI) and micronuclei (MN).

The results show (Table 1) that all experimental doses of fumagillin (25, 50 and 75 mg/kg b.w.) induced a significant decrease ($p<0.001$) in mitotic index (3.47 ± 0.04%, 3.17 ± 0.01% and 2.27 ± 0.02%, respectively) in comparison with the negative control (6.00 ± 0.01%). Moreover, a significant decrease ($p<0.001$) in MI was also observed in all experimental doses in comparison with the positive control (14.78 ± 0.09).

Table 1. Mitotic index in bone marrow cells treated with fumagillin

Treatment	Doses	Mitotic index (%)		
		Min - Max	Mean ± SE	SD
Water-sugar syrup (negative control)	1 water : 1 sugar	5.95 – 6.07	6.00 ± 0.01	0.04
Fumagillin	25 mg/kg b.w.	3.33 – 3.66	3.47 ± 0.04***	0.12
Fumagillin	50 mg/kg b.w.	3.13 – 3.19	3.17 ± 0.01***	0.02
Fumagillin	75 mg/kg b.w.	2.19 – 2.34	2.27 ± 0.02***	0.07
Cyclophosphamide (positive control)	40 mg/kg b.w	14.28 – 15.06	14.78 ± 0.09***	0.26

SE, standard error; SD, standard deviation;
***$p<0.001$, significantly different from the negative control (LSD-test)

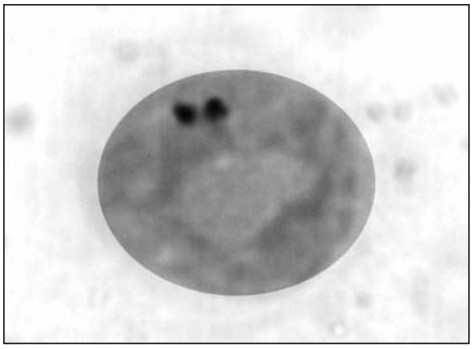

Figure 1. Microphotograph shows one polychromatic erythrocyte with two micronuclei of BALB/c strain mice induced by fumagillin in a dose of 25 mg/kg b.w.

Administration of fumagillin showed an ability to induce micronuclei (MN) (Figure 1) in polychromatic erythrocytes (PCE) of bone marrow of BALB/c mice (Table 2). Statistical analysis demonstrated a significant increase ($p<0.001$) in micronuclei induction (4.98 ± 0.35, 8.45 ± 0.57 and 12.02 ± 0.37) at all three doses of fumagillin (25, 50 and 75 mg/kg b.w., respectively) over the negative control (1.04 ± 0.28). In addition, the results demonstrated a dose-response relationship between exposure to fumagillin and frequency of MN in PCE.

Table 2. Frequency of micronucleated erythrocytes in bone marrow cells of control groups and experimental groups of BALB/c strain mice treated with increasing doses of fumagillin

Treatment	Concentration	Mean value No. of MN	SD	Xk
Negative control	water:sugar – 1:1	1.04	0.28	4.76
Positive control	40 mg/kg b.w.	21.86***	0.71	100
Fumagillin	25 mg/kg b.w.	4.98***	0.35	22.78
Fumagillin	50 mg/kg b.w.	8.45***	0.57	38.65
Fumagillin	75 mg/kg b.w.	12.02***	0.37	54.99

MN, micronuclei; ***$p<0.001$ (ANOVA, Student's *t* test); SD, standard deviation; Xk, percentage of mean value of MN of positive control group

DISCUSSION

Fumagillin is the only chemical registered for the treatment of *Nosema* infections in honey bees, whilst residues of fumagillin from honey, due to its high stability in honey (Assil and Sporns, 1991) and other food based on honey bee products, can easily reach consumers (including children, adolescents, convalescents, chronic patients and the elderly) (Stanimirovic *et al*., 1999; Stevanovic *et al*., 2000). Furthermore, topical fumagillin is suggested for treatment of ocular infections caused by microsporidia (Roseger *et al*., 1993; Wilkins *et al*., 1994); whilst recent investigations support the use of oral fumagillin as an effective treatment for chronic *Enterocytozoon bieneusi* infection in immunocompomised patients (Molina *et al*., 2000, 2002; Conteas *et al*., 2000). All the above mentioned indicates the need to evaluate genotoxic properties of fumagillin. The available data referring to genotoxic effects of fumagillin (dicyclohexylamine) obtained by *in vitro* investigations are equivocal, i.e. either positive (Stoltz *et al*., 1970; Stanimirovic *et al*., 1999; Stevanovic *et al*., 2000) or negative (Purchase *et al*., 1978; Mortelmans *et al*., 1986; Heil *et al*., 1996) and there are no references regarding genotoxic effects of fumagillin *in vivo* (Toxicological Evaluation, 2000).

In our *in vivo* study the mitotic index (MI) and micronuclei (MN) were monitored in bone marrow cells of BALB/c strain mice treated with selected doses of fumagillin (25, 50 and 75 mg/kg b.w.). The results show that all experimental

doses of fumagillin induced a significant decrease ($p<0.001$) in MI (Table 1) in comparison with the negative control. Moreover, a significant decrease ($p<0.001$) in MI was also observed in all experimental doses in comparison with the positive control (Table 1). These results are in accordance with the findings of other authors considering the antiproliferative effects (antiangiogenic effects) of fumagillin. Ingber *et al*. (1990) were the first to report that the fungal metabolite fumagillin potently suppresses angiogenesis due to its strong inhibition of endothelial cell proliferation *in vitro* and tumor-induced neovascularization *in vivo*. Wang *et al*. (2000) revealed that fumagillin inhibits human umbilical vein endothelial cells (HUVEC) proliferation at low nM concentration, and has partial or no effect on non-endothelial cells at concentrations up to 1 μM. The molecular target of fumagillin and its analogue TNP-470 is methionine aminopeptidase-2 (MetAP-2) (Sin *et al*., 1997; Griffith *et al*., 1998; Liu *et al*., 1998). Fumagillin binds MetAP-2 on His-231, thus inactivating the enzyme. MetAP-2 removes the N-terminal methionine from most proteins involved in cell cycle regulation as a part of the translocation process, hence its inhibition results in the cell cycle arrest and apoptosis (Fardis *et al*., 2003). These findings provided a starting point for the rational design of novel fumagillin analogues with fewer side-effects than fumagillin (Fardis *et al*., 2003).

Keezer *et al*. (2003) reported that fumagillin and its synthetic derivative, TNP-470, target the endothelial cell cytoskeleton through modification of the phosphorylation state and subcellular localization of cofilin and hsp27, two proteins involved in actin cytoskeleton and focal adhesion of endothelial cells. Mazzanti *et al*. (2004) confirmed that the antiangiogenic agent fumagillin is specific for the inhibition of endothelial cell proliferation. Moreover, these authors investigated the early effects of the antiangiogenic agents, endostatin and fumagillin, on the gene expression profiles in HUVEC by microarray analysis in order to elucidate pathways common to the effects of these agents. Their results supported the notion that genes DOC1, KLF4, and TC1 are specific for the endothelial cells response to endostatin and fumagillin, therefore they suggested that these genes might be involved in the initial pathways of angiogenesis inhibition. Nevertheless, Mazzanti *et al*. (2004) mentioned that further studies should be necessary to clarify these early mechanisms and present a better understanding of the functions of these genes.

Some reports indicate that the synthetic analogue of fumagillin TNP-470 inhibits tumor growth *in vivo*, but without an unambiguous corresponding decrease in tumor vasculature (Katzenstein *et al*., 1999; Shusterman *et al*., 2001; Morales *et al*., 2002). In addition, TNP-470 initial clinical trials produced inconclusive results, without a clear evidence of either reduced tumor growth or vascularity (Logothetis *et al*., 2001). In particular, alterations in serum levels of surrogate markers for antiendothelial activity were not detected (Logothetis *et al*., 2001). Moreover, Huang *et al*. (2004) detected a unique pattern of remodelling of tumor vasculature after TNP-470 administration, characterized by rapid and striking proliferation of microvessels. In addition, TNP-470 rapidly induced tumor cell apoptosis. These findings suggest that TNP-470 does not have antitumor

effects due to antiangiogenic activity, but rather due to a direct action on tumor cells (Huang *et al*., 2004).

Recently, after observations of short-term toxicity of TNP-470 in the B16F10 murine melanoma cell line *in vitro* and investigations of the mechanism of action, Okroj *et al*. (2005) concluded that TNP-470 could induce intracellular generation of reactive oxygen species (ROS), which act toxically inside B16F10 cells. One may suggest that this novel activity of TNP-470 might be beneficial in some cases, but it could also be responsible for some undesirable side-effects.

Stimulation of angiogenesis may have a place in the treatment of cardiovascular disease. Reestablishing blood flow to ischemic tissues through angiogenesis may provide a biologic "bypass" for patients with ischemic heart disease or peripheral vascular disease (Gibaldi, 1998). Thus, the possibility of fumagillin and its residues to reach consumers and patients through honey (Stanimirovic *et al*., 1999; Stevanovic *et al*., 2000) would be a disturbing factor of the specified beneficial effects of angiogenesis due to antiangiogenic activities of fumagillin and its analogues. Taking this into account, as well as the fact that fumagillin is suggested for topical treatment of ocular infection caused by microsporidia (Roseger *et al*., 1993; Wilkins *et al*., 1994) and oral treatment of chronic *Enterocytozoon bieneusi* infection in patients with AIDS and other types of immunodeficiency (Molina *et al*., 2000, 2002; Conteas *et al*., 2000) and our results which show a decrease in the MI in the bone marrow cells of BALB/c mice raise a question: what would happen if the named consumers had ischemic heart disease or peripheral vascular disease?

Furthermore, *in vitro* investigations of dicyclohexylamine gave no indication of its genotoxic potential in the Salmonella/microsome assay (Purchase *et al*., 1978; Mortelmans *et al*., 1986). Moreover, investigations of the DNA-damaging effect of dicyclohexylamine in the UMU test and in the DNA synthesis inhibition test in HeLa S3 cells, gave no indication that dicyclohexylamine had a damaging effect on DNA (Heil *et al*., 1996), which is rather surprising considering that fumagillin has primarily two epoxide structures capable of alkylating proteins involved in the packaging of DNA (Birch and Hussain, 1969) thereby establishing conditions for DNA damage. However, in cytogenetic studies carried out by Stoltz *et al*. (1970) dicyclohexylamine sulfate showed a concentration-depended increase in the aberration rate from approx. 6% in the controls to approx. 16% in the experimental groups. Besides, there are no references regarding the ability of dicyclohexylamine to induce micronuclei.

In addition, the National Toxicology Program (National Toxicology Program 2006a,b) reports a positive genotoxic effect in *Salmonella* test for dicyclohexylamine nitrite (National Toxicology Program 2006a) and negative for dicyclohexylamine (National Toxicology Program 2006b). Besides, there are references about the exerted genotoxic effects of secondary metabolites (gliotoxin and verruculogen) of *Aspergillus fumigatus*, the fungus that produces fumagillin, as well (Golden *et al*., 1998; Nieminen *et al*., 2002; Sabater-Vilar *et al*., 2003).

Our *in vivo* investigations of genotoxic effects of fumagillin, using the micronucleus (MN) assay, showed that all tested doses significantly ($p<0.001$)

increased the frequency of MN in PCE. The results clearly show a correlation between dose and effect, i.e. increased doses of fumagillin induced an increase in the frequency of MN (Table 2; Figure 1). This dose effect relationship points to the genotoxic properties of fumagillin. There is no possibility for direct comparasion of these results since data regarding the ability of fumagillin to induce MN are lacking. Nevertheless, our results are in accordance with the published findings, considering the ability of other drugs and pesticides to increase the frequency of MN in PCE (Çelik *et al*. 2003; Bajic *et al*., 2004; Andrighetti-Fröhner *et al*., 2006; Itoh *et al*., 2006).

Finally, our results related to the decrease in MI, and the increases in MN frequency induced by fumagillin lead to the conclusion that fumagillin residues in honey, even at doses as tested in our study, if they are consumed by the elderly, chronic patients and convalescents, could have an additional harmful influence on their health condition. In addition, there could be an effect on the absorption of drugs already used as ageing and various xenobiotics reduce the capacity for drug-metabolising enzymes (Ingelman-Sundberg, 2001; Stanimirovic *et al*., 2005).

In order to insure the safety of consumers, it is necessary to educate beekeepers regard the use of fumagillin, even more as fumagillin has antiproliferative properties as well as the ability to increase the frequency of MN in PCE. A similar caution should be taken with patients treated with fumagillin against microsporidia.

Moreover, additional studies of the adverse effects of fumagillin should be undertaken in order to provide all the necessary data to define an MRL for this substance; our results should not be disregarded in any case.

ACKNOWLEDGEMENT:
These studies were supported by the Serbian Ministry of Science and Environmental Protection, Grant No. 143022 (Ecophysiological and genetic investigations of domestic animals and bees for the purpose of increasing reproductive traits and disease resistance).

Address for correspondence:
Stevanović Jevrosima
Department of Biology,
Faculty of Veterinary Medicine,
University of Belgrade,
Bulevar oslobođenja 18
11000 Belgrade, Serbia
e-mail: rocky@vet.bg.ac.yu

REFERENCES

1. *Adler ID,* 1984, Cytogenetic tests in mammals, In: Venitt S, Parry JM, editors, Mutagenicity Testing: A Practical Approach, IRI, Oxford, 275–306.
2. *Anderson D, Bishop JB, Garner RC, Selby PB,* 1995, Cyclophosphamide: review of its mutagenicity for an assessment of potential germ cell risk, *Mutat Res*, 330, 115-8.
3. *Andrighetti-Fröhner CR, Kratz JM, Antonio RV, Creczynski-Pasa TB, Barardi CRM,* 2006, Simões CMO, *In vitro* testing for genotoxicity of violacein assessed by Comet and Micronucleus assays, *Mutat Res*, 603, 97-103.

4. *Assil HI, Sporns P,* 1991, ELISA and HPLC methods for analysis of fumagillin and its decomposition products in honey, *J Agric Food Chem* 39, 2206-13.
5. *Bailey L,* 1953, Effect of fumagillin upon *Nosema apis* (Zander), *Nature*, 171, 212-3.
6. *Bajic V, Stanimirovic Z, Stevanovic J*, 2004, Genotoxicity potential of 8-Cl-Cyclic Adenosine Monophosphate Assessed with Cytogenetic Test *In Vivo*, *Arch Med Res*, 35, 209-14.
7. *Birch AJ, Hussain SF,* 1969, Studies in relation to biosynthesis. Part 38. A preliminary study of fumagillin, *J Chem Soc*, 11, 1473-4.
8. *Çelik A, Mazmanci B, Çamlica Y, Askin A, Çömelekoglu Ü,* 2003, Cytogenetic effects of lambda-cyhalothrin on Wistar rat bone marrow, *Mutat Res*, 539, 91-97.
9. *Conteas CN, Berlin OG, Ash LR, Pruthi JS,* 2000, Therapy for human gastrointestinal microsporidiosis, *Am J Trop Med Hyg*, 63, 121-7.
10. *Didier ES,* 2005, Microsporidiosis: an emerging and opportunistic infection in humans and animals, *Acta Trop* 94, 61-76.
11. *El-Matbouli M, Hoffmann RW,* 1991, Prevention of experimentally induced whirling disease in rainbow trout *Oncorhynchus mykiss* by Fumagillin, *Dis Aquat Organ* 10, 109-13.
12. EMEA-The European Agency for the Evaluation of Medicinal Products, Veterinary Medicines and Information Technology, EMEA/CVMP/411/00-FINAL, Committee for Veterinary Medicinal Products, Update of the Position Paper on Availability of Veterinary Medicines agreed on 21 June 2000.
13. *Fardis M, Pyun HJ, Tario J, Jin H, Kim CU et al,* 2003, Design, synthesis and evaluation of a series of novel fumagillin analogues, *Bioorg Med Chem*, 11, 5051-8.
14. FDA Approved Animal Drug Products Online Database System Drug Product Abstract, http://meds.glowingpets.com/veterinary/2dawnk_9-23.html. Chapter 1.2 - Tradenames and Sponsors - Arranged by NADA Number: 009-252, Fumidil B, Mid-Continent Agrimarketing, Inc. 009-339, Carafen Cough, http://www.census.gov/foreign-trade/schedules/b/2005/alpha-f.html.
15. *Furgala B,* 1962, Residual fumagillin activity in sugar syrup stored by wintering honeybee colonies, *J Apic Res* 1, 35-7.
16. *Gibaldi M,* 1998, Regulating angiogenesis: A new therapeutic strategy, *J Clin Pharmacol*, 38, 898-903.
17. *Gilbert MA, Granath WO Jr,* 2003, Whirling disease and salmonid fish: life cycle, biology, and disease, *J Parasitol*, 89, 658-67.
18. *Griffith EC, Su Z, Niwayama S, Ramsay CA, Chang YH et al,* 1998, Molecular recognition of angiogenesis inhibitors fumagillin and ovalicin by methionine aminopeptidase 2, *Proc Natl Acad Sci USA*, 95, 15183-8.
19. *Golden MC, Hahm SJ, Elessar RE, Saksonov S, Steinberg JJ,* 1998, DNA damage by gliotoxin from *Aspergillus fumigatus*, *Mycoses*, 41, 97-104.
20. *Hedrick RP, Groff JM, McDowell T,* 1988, Oral administration of Fumagillin DCH protects chinook salmon *Oncorhynchus tshawytscha* from experimentally-induced proliferative kidney disease, *Dis Aquat Organ*, 4, 165-8.
21. *Heil J, Reifferscheid G, Waldmann P, Leyhausen G, Geurtsen W,* 1996, Genotoxicity of dental materials, *Mutat Res*, 368, 181-94.
22. *Huang J, Frischer JS, New T, Kim ES, Serur A et al,* 2004, TNP-470 promotes initial vascular sprouting in xenograft tumors, *Mol Cancer Ther*, 3, 335-43.
23. *Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T et al,* 1990, Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth, *Nature*, 348, 555-7.
24. *Ingelman-Sundberg M*, 2001, Genetic variability in susceptibility and response to toxicants, *Toxicol Lett*, 120, 259-68.
25. *Itoh T, Mitsumori K, Kawaguchi S, Sasaki YF,* 2006, Genotoxic potential of quinolone antimicrobials in the in vitro comet assay and micronucleus test, *Mutat Res*, 603, 135-44.
26. *Karagouni E, Athanassopoulou F, Lytra A, Komis C, Dotsika E*, 2005, Antiparasitic and immunomodulatory effect of innovative treatments against *Myxobolus sp*. infection in *Diplodus puntazzo*, *Vet Parasitol*, 134, 215-28.

27. *Katzenstein HM, Rademaker AW, Senger C, Salwen HR, Nguyen NN et al,* 1999, Effectiveness of the angiogenesis inhibitor TNP-470 in reducing the growth of human neuroblastoma in nude mice inversely correlates with tumor burden, *Clin Cancer Res*, 5, 4273-8.
28. *Katznelson H, Jamieson CA*, 1952, Control of *Nosema* disease of honey-bees with fumagillin, *Science*, 115, 70-1.
29. *Keezer SM, Ivie SE, Krutzsch HC, Tandle A, Libutti SK et al*, 2003, Angiogenesis inhibitors target the endothelial cell cytoskeleton through altered regulation of heat shock protein 27 and cofilin, *Cancer Res*, 63, 6405-12.
30. *Kent ML, Dawe SC*, 1994, Efficacy of fumagillin Dch# against experimentally-induced *Loma salmonae* (Microsporea) infections in chinook salmon *Oncorhynchus tshawytscha*, *Dis Aquat Organ*, 20, 231-3.
31. *Killough JH, Magill GB, Smith RC*, 1952, The treatment of amebiasis with fumagillin, *Science*, 115, 71-2.
32. *Kurebayashi J, Kurosumi M, Dickson RB, Sonoo H*, 1994, Angiogenesis inhibitor *O*-(chloroacetyl-carbamoyl) fumagillol (TNP-470) inhibits tumor angiogenesis, growth and spontaneous metastasis of MKL-4 human breast cancer cells in female athymic nude mice, *Breast Cancer*, 1, 109-15.
33. *Le Gouvello R, Pobel T, Richards RH, Gould C,* 1999, Field efficacy of a 10-day treatment of fumagillin against proliferative kidney disease in rainbow trout *Oncorhynchus mykiss*, *Aquaculture*, 171, 27-40.
34. *Liu S, Widom J, Kemp CW, Crews CM, Clardy J,* 1998, Structure of human methionine aminopeptidase-2 complexed with fumagillin, *Science*, 282, 1324-7.
35. *Logothetis CJ, Wu KK, Finn LD, Daliani D, Figg W et al,* 2001, Phase I trial of the angiogenesis inhibitor TNP-470 for progressive androgen-independent prostate cancer, *Clin Cancer Res*, 7, 1198-203.
36. *Mazzanti CM, Tandle A, Lorang D, Costouros N, Roberts D et al,* 2004, Early genetic mechanisms underlying the inhibitory effects of endostatin and fumagillin on human endothelial cells, *Genome Res*, 14, 1585-93.
37. *McCowen MC, Callender ME, Lawlis JF Jr,* 1951, Fumagillin (H-3), a new antibiotic with amebicidal properties, *Science*, 113, 202-3.
38. *Molina JM, Goguel J, Sarfati C, Michiels JF, Desportes-Livage I* et al, 2000, Trial of oral fumagillin for the treatment of intestinal microsporidiosis in patients with HIV infection, *AIDS*, 14, 1341-8.
39. *Molina JM, Tourneur M, Sarfati C, Chevret S, de Gouvello A et al,* 2002, Fumagillin treatment of intestinal microsporidiosis, *N Engl J Med*, 346, 1963-9.
40. *Morales C, Zurita M, Vaquero J,* 2002, Antitumor effect of TNP-470 is not associated to decrease of angiogenesis in an experimental malignant neuroectodermic tumor, *J Neurooncol*, 58, 131-6.
41. *Morris DJ, Adams A, Smith P, Richards RH,* 2003, Effects of oral treatment with TNP-470 on rainbow trout (*Oncorhynchus mykiss*) infected with *Tetracapsuloides bryosalmonae* (Malacosporea), the causative agent of proliferative kidney disease, *Aquaculture*, 221, 51-64.
42. *Mortelmans K, Haworth S, Lawlor T, Speck W, Tainer B et al,* 1986, Salmonella mutagenicity tests: II. Results from testing of 270 chemicals, *Environ Mutagen*, 8, Suppl 7, 1-119.
43. National Toxicology Program (2006a), http://ntp.niehs.nih.gov/index.cfm?objectid=6DE0B88C-F1F6-975E-7A19428A3AD94D85
44. National Toxicology Program (2006 b) http://ntp.niehs.nih.gov/index.cfm?objectid=6DE0E43F-F1F6-975E-7B0FA8000D056A43
45. *Nieminen SM, Maki-Paakkanen J, Hirvonen MR, Roponen M, von Wright A,* 2002, Genotoxicity of gliotoxin, a secondary metabolite of *Aspergillus fumigatus*, in a battery of short-term test systems, *Mutat Res*, 520, 161-70.
46. *Okroj M, Kamysz W, Slominska EM, Mysliwski A, Bigda J,* 2005, A novel mechanism of action of the fumagillin analog, TNP-470, in the B16F10 murine melanoma cell line. *Anticancer Drugs*, 16, 817-23.
47. *Purchase IFH, Longstaff E, Ashby J, Styles JA, Anderson D et al,* 1978, An evaluation of 6 short-term tests for detecting organic chemical carcinogens, *Br J Cancer*, 37, 873-959.

48. *Roserger DF, Serdaravic ON, Evlandson RA, Bryan RT, Schwartz DA et al*, 1993, Successful treatment of microsporidial keratoconjunctivitis with topical fumagillin in a patient with AIDS, *Cornea*, 12, 261-5.
49. *Ruddy JM, Marc C, Majumdar SK*, 2000, *In vitro* cytotoxic evaluation of fumagillin, a potent anti-angiogenic chemical, on mouse and human cancer cell lines. *Adv Pharmacol Toxicol*, 1, 1-11.
50. *Sabater-Vilar M, Nijmeijer S, Fink-Gremmels J,* 2003, Genotoxicity assessment of five tremorgenic mycotoxins (fumitremorgen B, paxilline, penitrem A, verruculogen, and verrucosidin) produced by molds isolated from fermented meats, *J Food Prot*, 66, 2123–9.
51. *Schmid W*, 1975, The micronucleus test, *Mutat Res*, 31, 9-15.
52. *Shadduck JA,* 1980, Effect of fumagillin on *in vitro* multiplication of *Encephalitozoon cuniculi*, *J Protozool* 27, 202–28.
53. *Shusterman S, Grupp SA, Barr R, Carpentieri D, Zhao H et al,* 2001, The angiogenesis inhibitor TNP-470 effectively inhibits human neuroblastoma xenograft growth, especially in the setting of subclinical disease, *Clin Cancer Res*, 7, 977–84.
54. *Sin N, Meng L, Wang MQ, Wen JJ, Bornmann WG et al,* 1997, The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. *Proc Natl Acad Sci USA*, 94, 6099–103.
55. *Stanimirovic Z, Stevanovic J, Pejovic D,* 1999, Analysis of genotoxic effects of fumagillin-ET, In: Proceedings of the 29th Annual Meeting of the European Environmental Mutagen Society-EEMS-99, vol. 85, Copenhagen, 44-5.
56. *Stanimirovic Z, Stevanovic J, Jovanovic S, Andjelkovic M,* 2005, Evaluation of genotoxic effects of Apitol® (cymiazole hydrochloride) *in vitro* by measurement of sister chromatid exchange, *Mutat Res*, 588, 152-7.
57. *Stevanovic J, Stanimirovic Z, Djelic N, Djurkovic Snezana,* 2000, Is the application of fumagillin in nosemosis treatment acceptable from a genotoxicological point of view. In: Trailovic D, Lazarevic M, editors, Proceedings of the 2nd Symposium in Animal Clinical Pathology and Therapy *Clinica Veterinaria*$_{2000}$, June 12-16, Budva, 227-30.
58. *Stoltz DR, Khera KS, Bendall R, Gunner SW,* 1970, Cytogenetic studies with cyclamate and related compounds, *Science*, 167, 1501-2.
59. *Tanaka T, Konno H, Matsuda I, Nakamura S, Baba S,* 1995, Prevention of hepatic metastasis of human colon cancer by angiogenesis inhibitor TNP-470, *Cancer Res*, 55, 836-9.
60. Toxicological Evaluation, last updated 11/2000, Dicyclohexylamine, CAS No. 101-83-7. BG Chemie, Berufsgenossenschaft der chemischen Industrie, No. 212.
61. *Turk BE, Su Z, Liu JO,* 1998, Synthetic analogues of TNP-470 and ovalicin reveal a common molecular basis for inhibition of angiogenesis and immunosuppression, *Bioorgan Med Chem*, 6, 1163-9.
62. *Wang J, Lou P, Henkin J,* 2000 Selective inhibition of endothelial cell proliferation by fumagillin is not due to differential expression of methionine aminopeptidases, J. Cell. Biochem. 77, 465-73.
63. *Webster TC*, 1994, Fumagillin affects *Nosema apis* and honey bees (Hymenoptera: Apidae), *J Econ Entomol*, 87, 601-4.
64. *Wilkins JH, Joshi N, Margolis TP, Cevallos V, Dawson DR,* 1994, Microsporidial keratoconjunctivitis treated successfully with a short course of fumagillin, *Eye*, 8, 703-4.
65. *Yamaoka M, Yamamoto T, Ikeyama S, Sudo K, Fujita T,* 1993, Angiogenesis inhibitor TNP-470 (AGM-1470) potently inhibits the tumor growth of hormone-independent human breast and prostate carcinoma cell lines, *Cancer Res*, 53, 5233-6.
66. *Yanase T, Tamura M, Hujita K, Kodama S, Tanaka K,* 1993, Inhibitory effect of angiogenesis inhibitor TNP-470 on tumor growth and metastasis of human cell lines *in vitro* and *in vivo*, *Cancer Res*, 53, 2566-70.
67. *Yoshida T, Kaneko Y, Tsukamoto A, Han K, Ichinose M et al,* 1998, Suppression of hepatoma growth and angiogenesis by a fumagillin derivative TNP-470: Possible involvement of nitric oxide synthase, *Cancer Res*, 58, 3751-6.
68. *Zbinden M, Lass S, Refardt D, Hottinger J, Ebert D,* 2005, *Octosporea bayeri*: fumidil B inhibits vertical transmission in *Daphnia magna*, *Exp Parasitol* 109, 58-61.

69. *Zimonjic DB, Savkovic N, Andjelkovic M,* 1990, Genotoxic agents: the effects: the effects, principles and methodology of detection, Naucna knjiga, Beograd, 1-395.

PRAĆENJE MITOTSKOG INDEKSA I UČESTALOSTI MIKRONUKLEUSA U FUNKCIJI PROCENE GENOTOKSIČNOG POTENCIJALA FUMAGILINA (DICIKLOHEKSILAMINA) *IN VIVO*

STEVANOVIĆ JEVROSIMA, STANIMIROVIĆ Z, PEJIN IVANA i LAZAREVIĆ M

SADRŽAJ

Fumagilin (dicyclohexylamine) je prirodni antibiotik koga produkuje *Aspergillus fumigatus*. Upotrebljava se u veterinarskoj medicini protiv mikrosporidioza pčela i riba, ali i u humanoj medicini za tretman intestinalnih amebijaza, microsporidijalnih keratokonjunktivitisa i intestinalnih mikrosporidioza uzrokovanih sa *Enterocytozoon bieneusi* kod pacijenata obolelih od AIDS-a i drugih tipova imunodeficijencije. U ovom radu procenjivan je genotoksični potencijal fumagilina u ćelijama kostne srži miševa praćenjem mitotskog indeksa i učestalosti pojave mikronukleusa u polihromatofilnim eritrocitima. Fumagilinom su u 7-dnevnom tretmanu tretirani BALB/c miševi intragastrično u dozama 25, 50, 75 mg/kg TM, u intervalima od 24h. Kao negativna kontrola korišćen je vodeno-šećerni sirup, dok je ciklofosfamid (40 mg/kg TM) korišćen kao pozitivna kontrola. Sve eksperimentalne doze fumagilina su izazvale signifikantno smanjenje ($p<0.001$) mitotskog indeksa (3.47 ± 0.04%, 3.17 ± 0.01% i 2.27 ± 0.02%, respektivno) u poređenju sa negativnom kontrolom (6.00 ± 0.01%), kao i u odnosu na pozitivnu kontrolu (14.78 ± 0.09). Fumagilin je signifikantno ($p<0.001$) povećao frekvenciju pojave mikronukleusa (4.98 ± 0.35, 8.45 ± 0.57 i 12.02 ± 0.37, respektivno) u odnosu na negativnu kontrolu (1.04 ± 0.28). Ovi rezultati ukazuju da fumagilin (dicyclohexilamine) ispoljava antiproliferativni (citotoksični) i genotoksični potencijal u sisarskom *in vivo* testu.