Acta Veterinaria (Beograd), Vol. 54. No. 2-3, 239-246, 2004.

UDK 619:615.916

EVALUTION OF THE GENOTOXIC EFFECTS OF TIAMULIN S- *IN VIVO*

MARKOVIĆ BILJANA, STANIMIROVIĆ Z and DJELIĆ N

Department of Biology, Faculty of Veterinary Medicine, Belgrade

(Received 16. January 2004)

In this work the genotoxic effect of the antibiotic preparation Tiamulin S was investigated. The experiments were done in vivo using cytogenetic analysis on BALB/c mouse bone marrow cells. The occurrence of chromosomal alterations was monitored in bone marrow and germ cells. The clastogenic effect of Tiamulin S was monitored at three doses (0.01 ml/kg, 0.2 ml/kg and 0.4 ml/kg) through eight experimental cycles. The results obtained showed that Tiamulin S induces kariotype changes including both numerical (aneuploidies and polyploidies) and structural chromosomal aberrations (lesions, breaks and Robertsonian translocations). Thus, Tiamulin S exerts genotoxic potential. In addition, a clear dose-response effect was observed in this investigation.

Key words: Chromosomal aberrations, bone marrow, mouse, Tiamulin S, testicles

INTRODUCTION

The application of pharmacological preparations and their possible accumulation in living beings may lead to irreversible consequences at all organisational levels, including changes at the molecular and cellular level, as well as influences on such complex processes as development and changes of population genetic structure. From the genotoxicological point of view, it is particularly interesting to observe changes at the molecular genetic and cytogenetic level under the influence of pharmacological preparations (Turker, 2003). Bearing in mind many data in the literature concerning genotoxic effects of chemicals, there is serious concern about the use of antibiotics both in human and veterinary medicine. It should be emphasized that lack of adequate control in using of antibiotics and chemotherapeutics may lead to deleterious effects (Giri, *et.al*. 2003). In addition, there may be additional risks if treated animals or their products are subsequently used in human nutrition (Shima *et al*., 2003). Since there is a great number of pharmacological preparations used in veterinary medicine, it is important to perform adequate screening of agents suspected to be mutagens. There is a general tendency to use preparations with low toxicity and genotoxicity.

Genotoxic effects of the antibiotic tiamulin were previously shown in bacterial systems (Bock *et al*., 1982). Namely, this antibiotic interferes with the

functioning of ribosomal proteins. Moreover, a number of mutations has been detected on genes coding for ribosomal proteins. *In vitro* experiments have shown that tiamulin inhibits expression of human cytochrome cyt 3A cDNA, due to its direct binding to cytochromes (De Grone *et al*., 1995). Witkamp *et al*. (1995) revealed a strong inhibitory effect of tiamulin on cytochomes cyt 450, and a close relationship with complete inhibition of hydroxylation. In addition, tiamulin can accumulate in the cytoplasm and cause inhibition of protein synthesis in intracellular bacterial strains isolated from swine with proliferative enteropathy (Mc Orist *et al*., 1995). Antibiotics used in veterinary medicine, including tiamulin, represent a group of compounds whose biological effects are not thoroughly investigated at the genetic level (changes in structure, morphology and functioning of genetic material) in eukaryotic cells (FAO/WHO, 1995). The effect of Tiamulin S, at various doses on somatic and germ cells in inbred BALB/c mice was investigated here, *in vivo* using a cytogenetic test.

MATERIALS AND METHODS

Test substance. Tiamulin S (Hemofarm, Vrsac) contained 200 mg of tiamulin base per milliliter.

Treatment. Tiamulin was diluted in distilled water to achieve the doses needed for this investigation (0.01 ml/kg, 0.2 ml/kg i 0.4 ml/kg). Animals were treated intragastrally for three consecutive days. Two hours before sacrifice, the animals were treated with colchicine (i.p.) at a dose of 10 g/ml. The whole investigation was performed in eight experimental cycles. Each experimental group comprised 6 males. Untreated animals were used as controls.

In vivo cytogenetic test on somatic cells. Biological material for kariotype analysis was obtained from bone marrow according to Hsu and Patton (1969), slightly modified by Zimonjić *et al*. (1990). After the hypotonic treatment with 0.56% KCl, three repetitive cycles in methanol-acetic acid (3:1,v/v) were performed. Giemsa (Alkaloid, Skoplje) was used for chromosome staining.

In vivo cytogenetic test on germ cells. Metaphase figures were prepared from testicles of adult males according to Evans *et al*. (1964), with a slight modification by Zimonjić *et al* (1990). The cells were treated with 2,2% sodium citrate, centrifuged and resuspended with 1.1 % sodium citrate, followed by three repetitive cycles in methanol-acetic acid (3:1, v/v). Giemsa (Alkaloid, Skoplje) was used for chromosome staining.

Statistical analysis. The statistical significance of differences between the control and treated groups were determined by Student's *t*-test.

RESULTS

In animals treated for three days Tiamulin S caused changes in both chromosome number and morphology of bone marrow cells (Table 1 and Figure 1). As for the numerical aberrations we observed the following changes: in animals treated with 0.01 ml/kg there were 7.14% aneuploid and 0.52% polyploid

cells; at the 0.2 ml/kg dose there were 8.6% aneuploid and 0.55% polyploid cells; finally at the dose of 0.4 ml/kg there were 10.01% aneuploid and 1.2% polyploid cells. Structural aberrations were as follows: at the 0.01 ml/kg dose 1.9% had lesions, 1.3% had gaps and 0.24% showed Robertsonian translocations; at the dose of 0.2 ml/kg 4.1% of the cells had lesions, 1.73% had breaks and 1.16% showed Robertsonian translocations; at the dose of 0.4 ml/kg there were 5.46% with lesions, 2.35% with breaks and 2.02% with Robertsonian translocations. When presented as the total sum of overall changes, there was a clear dose-response relationship, because at 0.01 ml/kg b.w. there were 12.10% cells with changes, at 0.2 ml/kg b.w.16.14%, and at 0.4 ml/kg b.w. 21.04 % cells with chromosome changes.

Table 1. Cytogenetic parameters of bone marrow cells in control and Tiamulin S-treated mice

	Control		Tiamulin S 0.01ml/ b.w		Tiamulin S 0.2 ml/ b.w		Tiamulin S 0.4 ml/ b.w	
	X±SD	%	X±SD	%	X±SD	%	X±SD	%
Aneuploidy	1.21	0.17	49.96	7.14	60.21	8.60	70.13	10.01
Polyploidy	0	0	3.63	0.52	3.88	0.55	8.4	1.2
Lesions	1.83	0.26	20.33	1.9	28.73	4.1	38.23	5.46
Breaks	0.77	0.11	9.5	1.3	12.12	1.73	16.46	2.35
Robertsonian translocation	0	0	1.65	0.24	8.15	1.16	14.12	2.02
All cytogenetic changes	3.81	0.54	85.07	12.10	113.09	16.14	147.34	21.04

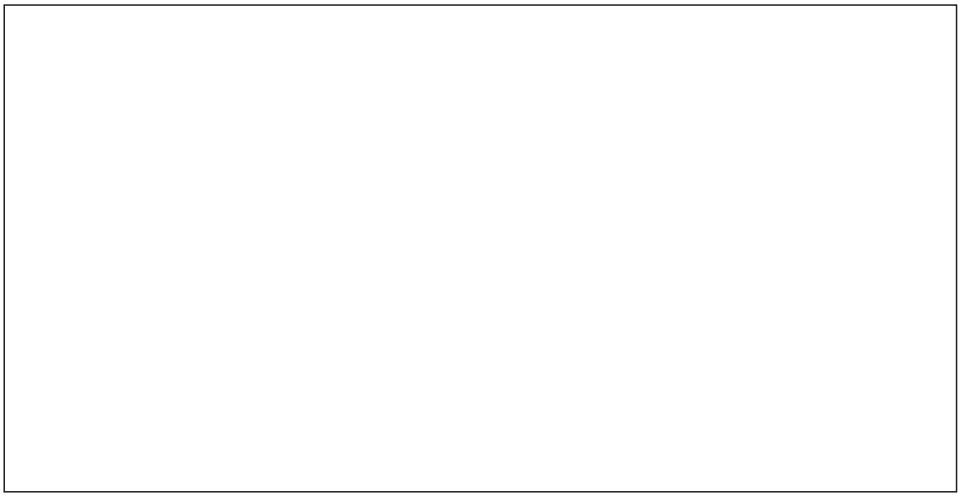

Figure 1. Percentages of cytogenetic changes in bone marrow cells in control and treated mice

Student *t*-test showed highly significant differences between the control and treated groups at all doses used in this experiment. (Table 2).

Table 2. Statistical analysis of the differences in overall cytogenetic changes in bone marrow cells between control and Tiamulin S-treated mice

	X ± SD	Tiamulin S 0.01 ml/ b.w.		Tiamulin S 0.2 ml/ b.w		Tiamulin S 0.4 ml/ b.w.	
		Sd	t	Sd	t	Sd	t
Control	3.82±0.92	0.78	104.15***	0.83	131.63***	0.87	165.01***
Tiamulin S 0.01ml/b.w	85.00±2.08			1.04	29.63***	1.08	57.7 ***
Tiamulin S 0.2 ml/tb.w	113.09±2.16					1.11	30.91***
Tiamulin S 0.4 ml/b.w.	147.34±2.29						

***p<0.001
d.f. = 14

Tiamulin S caused chromosome changes in germ cells as well. Both numerical and structural changes were observed (Table 3 and Figure 2.). The relative numbers of aberrant cells with polyploidies and aneuploidies were: at the dose of 0.01 ml/kg 7.76% aneuploid cells and 0.21% polyploid cells; at the dose of 0.2 ml/kg b.w. 9.28% of the cells were aneuploid and 0.41% polyploid; at the dose of 0.4 ml/kg b.w. 12.57% were aneuploid and 0.87% polyploid. As for the structural aberrations we obtained the following results: at the dose of 0.01 ml/kg there were

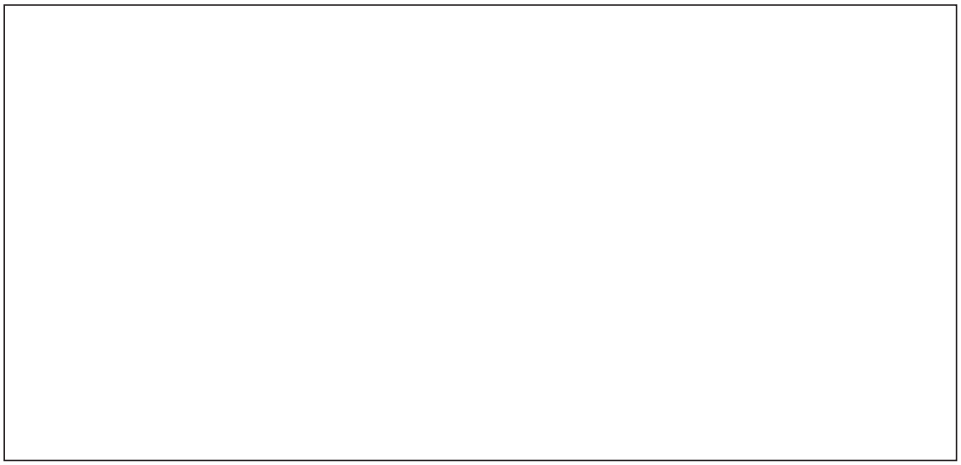

Figure 2. Percentages of cytogenetic changes in germ cells in control and treated mice

2.94% of cells with lesions, 1.03% with breaks and 0.49% of Robertsonian translocations; at the dose of 0.2 ml/kg there were 2.94% of cells with lesions, 1.98% with breaks and 0.93% with Robertsonian translocations: at 0.4ml/kg 4.83% with lesions, 2.44% with breaks and 1.7% with Robertsonian translocations. Overall cytogenetic changes were: at the dose of 0.01 ml/kg b.w.11.52%, at 0.2 ml/kg b.w.15.54% and at 0.4 ml/kg b.w.22.41%.

Table 3. Cytogenetic parameters in germ cells of control and Tiamulin S treated mice

	Control		Tiamulin S 0.01ml/ b.w		Tiamulin S 0.2 ml/ b.w		Tiamulin S 0.4 ml/ b.w	
	X ± SD	%	X ± SD	%	X ± SD	%	X ± SD	%
Aneuploidy	1.04	0.15	54.33	7.76	64.94	9.28	88	12.57
Polyploidy	0	0	1.48	0.21	2.88	0.41	6.08	0.87
Lesions	0.65	0.09	14.23	2.03	20.64	2.94	33.83	4.83
Breaks	0.29	0.04	7.20	1.03	13.89	1.98	17.11	2.44
Robertsonian translocation	0	0	3.46	0.49	6.5	0.93	11.9	1.70
All cytogenetic changes	1.98	0.28	80.70	11.52	108.85	15.54	156.92	22.41

Statistical analysis of the obtained results revealed that Tiamulin S at all doses applied caused highly significant alterations compared to the untreated controls. The same level of statistical significance was obtained for all treatments.

Table 4. Level of statistical significance of the differences in cytogenetic parameters of germ cells between control and Tiamulin S-treated mice

	X ± SD	Tiamulin S 0.01 ml/ b.w.		Tiamulin S 0.2 ml/ b.w		Tiamulin S 0.4 ml/ b.w.	
		Sd	t	Sd	t	Sd	t
Control	1.98 ± 0.75	0.63	125.16***	0.68	157.16***	1.08	143.38***
Tiamulin S 0.01ml/b.w	80.78 ± 1.59			0.84	33.36***	1.19	63.87***
Tiamulin S 0.2 ml/tb.w	108.85 ± 1.77					1.22	39.33***
Tiamulin S 0.4 ml/b.w.	156.92 ± 2.95						

***p<0.001
d.f. = 14

DISCUSSION

In vivo cytogenetic investigations in this work have shown that all doses (0.01 ml/kg, 0.2 ml/kg i 0.4ml/kg) of Tiamulin S induce chromosome alterations both in bone marrow and in germ cells of treated BALB/c mice. Intragastric treatment with Tiamulin S for a three days caused numerical (aneuploidies and polyploidies) and structural changes (lesions, breaks and Robertsonian translocations). According to Vaughan-Dellarco et. al. (1985), chemical preparations with genotoxic properties interact with the mitotic spindle and, therefore, can induce irregularities in chromosome segregation followed by the appearance of aneuploidies and/or polyploidies. In our experiments, induction of numeric aberrations with Tiamulin S exhibited a clear dose-response effect. The significant increase of cells with numeric aberrations points to the genotoxicity of Tiamulin S. We assume that Tiamulin S interacts with the mitotic spindle causing lagging of chromatids during anaphase. The other possibility is inactivation of the centriole and/or kinetochore.

One of the precise genotoxic endpoints is the appearance of structural chromosomal changes (lesions and breaks) and aberrations (Brogger,1982). Namely, it has been observed that lesions and breaks occur spontaneously, although with a very low frequency (Lilp, 1981; Irans, 1982). As for the structural changes in Tiamulin S-treated mice we observed that doses of 0.2 ml/kg and 0.4 ml/kg induced structural changes and, therefore, exhibit genotoxic action. The observed frequencies of structural chromosome changes significantly exceeded the spontaneous level established by Brogger (1982).

The appearance Robertsonian translocations, as a specific type of structural change, results from changes in centromeric regions susceptible to the effects of genotoxic agents. (Agulnik *et al*., 1990; Marković 1999; Marković *et al*., 2000). All doses of Tiamulin S caused Robertsonian translocations, thereby additionally demonstrating the genotoxic potential of the preparation.

In the light of our experimental data concerning the overall cytogenetic changes, we can conclude that there is a highly significant ($p<0.001$) difference between untreated and treated groups of mice, as well as a clear dose-dependence.

Our experimental data demonstrate that all doses of Tiamulin S exert both cytotoxic and genotoxic potential. Therefore, this antibiotic preparation should be replaced with a less toxic one, whenever possible.

Acknowledgements
These studies were supported by the Serbian Ministry of Science, Technology and Development, Grant No 1873.

Address for correspondence:
Dr Biljana Markovic
Department of Biology
Faculty of Veterinary Medicine
University of Belgrade
Bul. JNA 18
11000 Belgrade,
Serbia & Montenegro
E-mail: biolog@vet.bg.ac.yu

REFERENCES

1. *Agulnik S I, Agulnik A I, Gorlov I P,* 1990, Mejotičeskij drajv aberatnoj 1-oj hromozomi u domovoj misi, *Genetika*,6, 664-9.
2. *Bock A, Turnowsky F, Hogenauer G,* 1982, Tiamulin resistance mutations in *Escherichia coli, J Bacteriol*, 151, 1253-60.
3. *Brogger A,* 1982, Cytogenetic Cell, *Genet*, 33. 14-9.
4. *De Grone EM, Nijmeijer SM, Horbach GJ, Witkamp RF,* 1995, Tiamulin inhibits human CYP3A4 activity in an NIH/3T3 cell line stably expressing CYP3A4 cDNA, *Biiochem Pharmacol*, 22, 23-9.
5. *Giri S, Giri A, Sharma GD, Prasad SB,* 2003, Induction of sister chromatid exchanges by cypermethrin and carbosulfan in bone marrow cells of mice *in vivo, Mutagenesis*, 18, 53-8.
6. *Evans EP, Breckon G, Ford CE*, 1964, An air drying method for meiotic preparations from mammalian testes, *Cytogenetics*, 3, 289-94.
7. *FAO/WHO.* 1995, Forty-second Meeting of the Joint FAO/WHO Expert Committee on Food Additives, Evaluation of certain veterinary drug residues in food. WHO Technical Report Series No 851, 19 - 212. WHO, Geneva.
8. Hsu T C, Patton J I. 1969, Bone marrow preparations for chromosome studies. In Benirschke, V. (ed) Comparative Mammalian Cytogenetics, Springer Verlag, Berlin, Heidelberg, New York, p. 454-460.
9. *Irans DR*. 1985, Toxicology of the Blood and Bone Marrow, Raven Press New York.
10. *Jager LP, Vroomen LH*, 1990, Toxicological considerations in the evaluation of veterinary drugs, *Toxicology*, 15, 727-35.
11. *Lilp JG,* 1981, Cytol (USSR) XXIII, 10: 1174-79.
12. *Marković B,* 1999, Genotoksični efekat antibiotskih preparata Carbadox, Tiamulin S i Gastrogal 10. Doktorska disertacija, Fakultet veterinarske medicine, Univerzitet u Beogradu.
13. *Marković B, Stanimirović Z, Vučinić M, Čupić V,* 2000, Examination of Carbadox genotoxicity *in vitro* and *in vivo*, *Acta Vet*, Beograd 50: 387 – 96
14. *Mc Orist S, Mackie RA, Lawson GH,* 1995, Antimicrobial susceptibility of ileal symbiont intracellularis isolated from pigs with proliferative enteropathy, *J Clin Microbiol*, 33, 1314-17.
15. *Shima N, Hartford SA, Duffy T, Wilson LA, Schimenti KJ, Schimenti JC*, 2003, Phenotype-based identification of mouse chromosome instability mutants, *Genetics*, 163, 1031-40.
16. *Turker MS*, 2003, Autosomal mutation in somatic cells of the mouse, *Mutagenesis*, 18, 1-6.
17. *Vaughan-Dellarso VL, Marvournin KH, Tice RR*, 1985, Aneuploidy and health risk assessment: Future directions. Environ. Mutagen.
18. *Witkamp RF, Nijemeijer SM, Monshouwer M, Van Miert AS,* 1995, The antibiotic tiamulin a potent inducer and inhibitor of cytochrome P4503A via the formation of a stable metabolic intermediate complex. Studies in primary hepatocyte cultures and liver microsomes of the pig, *Drug Metab dispos*, 23, 542-7.
19. *Zimonjic D, Savkovic N, Andjelkovic M,* 1990, Genotoksicni agensi efekti, principi, i metodologija detekcija. Naučna knjiga, Beograd.

ISPITIVANJE GENOTOKSIČNOG EFEKTA TIAMULINA S *IN VIVO*

MARKOVIC BILJANA, STANIMIROVIC Z i DJELIC N

SADRŽAJ

U ovom radu su prikazani rezultati ispitivanja genotoksičnog efekta antibiotskog preparata Tiamulina S. Eksperiment *in vivo* obavljen je na laboratorijskim miševima BALB/c soja praćenjem uticaja intragastrične aplikacije Tiamulina S na nastanak hromozomskih promena u ćelijama kostne srži i semenika. Klastogeni efekat Tiamulina S praćen je u tri eksperimentalne doze (0,01 ml/kg, 0,2 ml/kg i 0,4 ml/kg) kroz osam eksperimentalnih ciklusa. Eksperimentalni rezultati pokazuju da ispitivani antibiotiski preparat Timulin S ima sposobnost promene, kariotipa ćelija kostne srži i testisa i indukcije numeričkih hromozomskih aberacija tipa aneuploidija i poliploidija, kao i strukturnih hromozomskih aberacija tipa lezija prekida i Robertsonovih translokacija. Dobijeni rezultati ukazuju da antibiotski preparat Tiamulin S ima određeni genotoksični potencijal. Takođe je uočena pozitivna korelacija između jačine primenjene doze testiranog antibiotskog preparata Tiamulina S i učestalosti numeričkih i strukturnih hromozomskih aberacija u ćelijama kostne srži i ćelijama testisa tretiranih životinja.