The aim of this study was to investigate the genotoxic effects of flunixin meglumine on mice peripheral lymphocytes by in vitro and in vivo/in vitro cytokinesis block micronucleus tests (CBMN). Flunixin meglumine was used at concentrations of 25, 50 and 100 µg/mL for the in vitro assay and 50, 75 and 100 mg/kg for the in vivo/in vitro assay. Mice were treated intraperitoneally twice with a 24 h interval and sacrificed 6 h after the last dose. Cardiac blood was taken and added to the cultures for the in vivo/in vitro test. 21 h after the addition of the test compound for the in vitro test, and after the initiation of incubation for in vivo/in vitro test cytokinesis was blocked with the addition of cytochalasin-B and 20 h later the cultures were harvested. In both test systems, a negative and a positive control mitomycin C (MMC) were also included.

The micronucleated binuclear cell (MNBN) frequencies increased after both treatments, however, the differences between the treated cells and the control groups were found to be statistically significant only for the in vitro treatment. The increase was in a dose-dependent manner, significant elevations of MNBN cell (p<0.05 and p<0.001) were observed at concentrations 50 and 100 µg/mL respectively. In addition reduction in cytokinesis-block proliferation index (CBPI) was observed in both treatments, indicating cytotoxicity of flunixin meglumine.

According to these results, flunixin meglumine is genotoxic in mice lymphocytes treated in vitro, but has not mutagenic activity in vivo under micronucleus (MN) test conditions.

Key words: cytochalasin-B, flunixin meglumine, micronucleus, mice, peripheral lymphocytes

INTRODUCTION

Flunixin meglumine is a non-steroid anti-inflammatory drug (NSAID) used in food-producing animals and indicated for the regulation of inflammation in endotoxemia and control of pyrexia (Buur et al., 2006). Flunixin meglumine is a...
cyclooxygenase inhibitor, blocks the biosynthesis of prostaglandins, which are believed to play a role in the development and progression of some forms of cancer (Jackman et al., 1994; Zha et al., 2004). Recent studies on prophylaxis and therapy of cancer established that NSAIDs have a cancer-chemopreventive action (Sheng et al., 1997; Shiff et al., 2003; Yao et al., 2003). However, there is no published data about the anticancerogenic potential of flunixin meglumine. On the other hand, based on carcinogenicity studies, the European Medicines Agency report (EMEA, 1999) demonstrated that flunixin meglumine is not carcinogenic. To our knowledge, the International Agency for Research on Cancer (IARC) has no available evaluation on this molecule (IARC). There is considerable evidence of a positive correlation between the carcinogenicity of substances in vivo and their mutagenicity in long-term studies with animals (Ashby and Tennant 1991; Bernauer et al., 2005). However, the genotoxicity profile of flunixin meglumine in short-term assays is somewhat equivocal due to the positive and negative results of the in vitro and in vivo genotoxicity tests. Committee on Mutagenicity (COM) reported that most of the mutagenicity data of flunixin meglumine were relatively old and had limitations (COM, 2001). Flunixin meglumine was not mutagenic in a limited number of Salmonella typhimurium strains in Ames test, unscheduled DNA synthesis assay in rat primary hepatocyte cultures and in vivo in a bone marrow micronucleus assay. In contrast flunixin meglumine had mutagenic potential in vitro in mouse lymphoma forward mutation assay, in vitro chromosome aberration assay in Chinese hamster ovary cells both in the absence and presence of S-9 metabolic activation and in a mitotic gene conversion assay in Saccharomyces cerevisiae (EMEA, 1999).

Flunixin meglumine is an ionic compound and in vivo dissociates rapidly in aqueous media at physiological pH to flunixin and meglumine. The primary purpose of meglumine is to act as a counter ion to keep flunixin soluble. Genotoxicity studies showed that there is no evidence to suggest flunixin to be mutagenic in vivo, in contrast meglumine mutagenicity results were inconsistent with some positive and negative results. The mutagenic activity seen in vitro with flunixin meglumine was believed to be due to the meglumine component (COM, 2003).

One of the test systems applied as a cytogenetic assay for biomonitoring and identification of genotoxic effects of physical and chemical agents was the cytokinesis block micronucleus (CBMN) technique. MN are chromosomal fragments or whole chromosomes that are not incorporated into daughter nuclei during mitosis because of chromosomal breakage or dysfunction of the mitotic apparatus, respectively. Cytochalasin-B, an inhibitor of actin polymerisation, prevents cytokinesis and produces binucleated (BN) cells which can be easily and accurately scored for MN following one cell cycle (MacLean-Fletcher and Pollard, 1980; Fenech and Morley, 1985; Fenech, 1993).

Due to the controversial findings of earlier studies on the genotoxic effects of flunixin meglumine, the present study was undertaken to obtain additional data on the cytogenetic activity of flunixin meglumine and to investigate whether flunixin meglumine is mutagenic in cultured mouse lymphocytes both in vivo and in vitro by using CBMN assay as the genetic endpoint.
MATERIALS AND METHODS

Animals

Experiments were performed using male CD-1 mice, aged 8-12 weeks and weighing 20-25 g, obtained from Pendik Veterinary Control and Research Institute (Turkey). The mice were housed in polypropylene cages and acclimatised for two weeks in the animal house, maintained at 23 ± 2 °C and humidity 50 ± 5% with a 12 h light/dark cycle. Feed and water were provided ad libitum. The experimental protocol was approved by Istanbul University Veterinary Faculty Ethic Committee (Regd, No. 2004/88).

Chemicals

Flunixin meglumine (2-[2-Methyl-3-(trifluoromethyl)phenyl]amino]-3-pyridinecarboxylic acid meglumine salt, Alke, Turkey, 99.5% purity) is soluble in physiological saline and was freshly prepared at concentrations 25, 50, 100 µg/mL for in vitro and 50, 75 and 100 mg/kg for in vivo/in vitro CBMN test before each experiment. Mitomycin C (MMC, Sigma, St. Louis, MO, USA), used as a positive control agent because of its clear response in the MN test, was dissolved in ultra-pure water to the concentration used, just prior to treatment.

In vitro micronucleus test

The doses for in vitro CBMN test were chosen according to the previous genotoxicity studies of flunixin meglumine (EMEA, 1999). CBMN was carried out using the Standard technique described by Fenech (2000; 2006) with slight modifications and the current OECD guideline (OECD, 2007). Briefly, blood samples were obtained by cardiac puncture with heparinised syringes from ether anaesthetised healthy mice. Whole blood (0.5 mL) was cultured in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 20% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA), antibiotics (penicillin 100 IU/mL and streptomycin 100 µg/mL) and 2% phytohaemagglutinin (PHA Sigma, St. Louis, MO, USA). The cultures were incubated for 62 h in a humidified environment with 5% CO₂ at 37°C. The test substance flunixin meglumine was added at three different concentrations 21 h after PHA stimulation. Cytochalasin B (6 µg/mL) (Cyt-B, Sigma, St. Louis, MO, USA) was added at 42 h post-culture initiation, to arrest cytokinesis of dividing cells. Negative (physiological saline) and positive controls (MMC 0.2 µg/mL) were run simultaneously and similarly with flunixin meglumine treated cultures. The treatment protocol is shown in Figure 1.

![Figure 1. Treatment protocol of in vitro micronucleus test](image-url)

**In vivo/in vitro micronucleus test**

Based on our preliminary experiment findings, doses of 50, 75 and 100 mg/kg b.w. of flunixin meglumine no observed mortality and toxicity signs were chosen for the *in vitro/in vivo* studies.

The experiments were performed as described by Moore *et al.*, (1995a,b), with application to lymphocytes. A total of 40 mice were divided into 5 groups. Flunixin meglumine (50, 75 and 100 mg/kg b.w.) was administered intraperitoneally twice with a 24-h interval at a volume of 10 mL/kg. In addition, a negative (physiological saline) and a positive (MMC, 2 mg/kg) control group were used to test the validity of the assay. Blood samples were obtained 6 h after the last treatment of flunixin meglumine and 48 h after physiological saline and MMC treatment. The test protocol was applied as described in *in vitro* assay. The treatment protocol is shown in Figure 2.

### MN assay

The cells were harvested by centrifugation (1000 rpm, 8 min), and were suspended in a hypotonic solution of 0.075M KCl at room temperature. Next, cells were recentrifuged (2000 rpm, 3 min) and fixed three times in cold methanol: acetic acid (6:1). Slides were prepared by dropping and air-drying. Slides were stained with 5% Giemsa (pH 6.8) in phosphate buffer for 10 min, washed in distilled water and dried at room temperature (Lee *et al.*, 1994 a,b).

For MN identification, all slides were analysed in accordance with Fenech (1997; 2000) using a Olympus CX31 microscope. The induction of MN was evaluated by scoring a total of 1000 binucleated (BN) cells with well-preserved cytoplasm at 1000 × magnification.

From the data of MN analysis, cytokinesis-block proliferation index (CBPI), which can be considered as an index of cell kinetics or average cell division, was calculated by classifying 1000 cells according to the number of nuclei for *in vitro* and *in vivo/in vitro* treatments as CBPI = (M1 + 2M2 + 3(M3 + M4) / N where M1-M4 represents the number of cells with 1 to 4 nuclei, respectively, and N is the total number of scored cells (Suralles *et al.*, 1995). This value indicates the number of cycles per cell during the period of exposure to cytochalasin.
Statistical Analysis

Statistical differences between the in vitro and in vitro/in vivo treatments and
the controls groups were tested by one-way analysis of variance (ANOVA)
followed by the Student–Newman–Keuls test using the “Instat” statistic computer
program. A difference in the mean values of p<0.05 or less was considered to be
statistically significant.

RESULTS

The frequency of binucleated cells with micronuclei (BNMN), cytotoxicity
index (CBPI) obtained after in vitro and in vivo/in vitro treatment with flunixin
meglumine is shown in Table 1 and Table 2, respectively. In vitro treatments in
concentrations ranging from 25, 50 and 100 µg/mL were found to induce BNMN
frequency with increasing concentrations of flunixin meglumine, reaching a
statistical significance at 50 and 100 µg/mL concentrations (p<0.05 and
p<0.001), respectively in the cytokinesis-blocked lymphocytes. Positive control
MMC yielded a depression of cell proliferation and positive response in MN
induction. The lowest concentration of flunixin meglumine (25 mg/kg) did not
show any significant effect. The reduction in the frequency of CBPI and % BN cells
with decreasing doses of the drug were observed in lymphocyte cultures,
indicating cytotoxicity of flunixin meglumine in both treatments.

MN analysis in in vivo/in vitro micronucleus assay showed that the flunixin
meglumine did not significantly increase the micronucleus frequency compared
with the negative control. A reduction in cell proliferation was found, reaching
statistical significance (p<0.001) at all test concentrations of flunixin meglumine
compared to the control group.

DISCUSSION

The use of drugs in food-producing animals can lead to potentially harmful
residues in edible products harvested from these animals. A risk assessment
which offers a formal approach to the evaluation of the safety of veterinary drug
residues is an essential component of the regulatory approval process for
products containing these drugs. Genotoxic activity has an impact on the risk
assessment of a veterinary drug (Gehring et al., 2006).

Despite the expansive use of flunixin meglumine as a non-steroid anti-
inflammatory drug, information on its toxicology is still incomplete. Since the
results of earlier studies on evaluation of the genotoxicological profile of flunixin
meglumine were contradictory and inconclusive (COM, 2005), this investigation
was conducted to evaluate whether flunixin meglumine is capable of changing a
normal cell cycle progression of mouse lymphocytes treated in vitro and in vivo/in
vitro by analysing the cytogenetic endpoint MN. Our in vitro experimental results
demonstrated a significant, partly dose-dependent increase of micronuclei at
concentrations of 50 and 100 µg/mL flunixin meglumine. Previously, flunixin
meglumine demonstrated clastogenic activity in Chinese hamster ovary cells
Table 1. Induction of micronuclei and CBPI values in mice lymphocytes treated with flunixin meglumine in vitro

<table>
<thead>
<tr>
<th>Administered compound and dose</th>
<th>No of mice</th>
<th>The total number of nucleated cells scored</th>
<th>The total number of binucleated cells scored</th>
<th>The total number of binucleated cells with micronuclei</th>
<th>Percentage of binucleated cells with micronuclei</th>
<th>Binucleated cells with micronuclei BNMN frequency (mean %±SD)</th>
<th>CBPI²</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS (NC)¹</td>
<td>8</td>
<td>18 691</td>
<td>8000</td>
<td>22</td>
<td>42.8</td>
<td>2.75±1.28</td>
<td>1.44±0.013</td>
</tr>
<tr>
<td>Flunixin meg. (25 µg/mL)</td>
<td>8</td>
<td>21 680</td>
<td>8000</td>
<td>30</td>
<td>36.9</td>
<td>3.75±1.04</td>
<td>1.38±0.009***</td>
</tr>
<tr>
<td>Flunixin meg. (50 µg/mL)</td>
<td>8</td>
<td>31 008</td>
<td>8000</td>
<td>47</td>
<td>25.8</td>
<td>5.90±1.46*</td>
<td>1.27±0.008***</td>
</tr>
<tr>
<td>Flunixin meg. (100 µg/mL)</td>
<td>8</td>
<td>37 037</td>
<td>8000</td>
<td>64</td>
<td>21.6</td>
<td>8±1.31***</td>
<td>1.21±0.011***</td>
</tr>
<tr>
<td>MMC (PC)¹ (0.2 µg/mL)</td>
<td>8</td>
<td>35 635</td>
<td>8000</td>
<td>395</td>
<td>22.45</td>
<td>49±2.33***</td>
<td>1.36±0.014***</td>
</tr>
</tbody>
</table>

¹ (NC) Negative control. (PC) Positive control
² (CBPI) Cytokinesis-block proliferation index; by counting 1000 cells and calculated according to the formulation.
CBPI = (MoN cell count + 2 x BN cell count + 3 x PN cell count) / total cell count
* p<0.05, *** p<0.001 (Compared with negative control group)
**Table 2. Induction of micronuclei and CBPI values in mice lymphocytes treated with flunixin meglumine in vivo/in vitro**

<table>
<thead>
<tr>
<th>Administered compound and dose</th>
<th>No of mice</th>
<th>The total number of nucleated cells scored</th>
<th>The total number of binucleated cells scored</th>
<th>The total number of binucleated cells with micronuclei</th>
<th>Percentage of binucleated cells</th>
<th>Binucleated cells with micronuclei BNMN frequency (mean % ±SD)</th>
<th>CBPI²</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS (NC)¹</td>
<td>8</td>
<td>14774</td>
<td>8000</td>
<td>33</td>
<td>54.15</td>
<td>4.1±1.2</td>
<td>1.60±0.029</td>
</tr>
<tr>
<td>Flunixin meg. (25 µg/ml)</td>
<td>8</td>
<td>17730</td>
<td>8000</td>
<td>39</td>
<td>45.12</td>
<td>5.1±0.1</td>
<td>1.49±0.023***</td>
</tr>
<tr>
<td>Flunixin meg. (50 µg/ml)</td>
<td>8</td>
<td>18757</td>
<td>8000</td>
<td>45</td>
<td>42.65</td>
<td>5.6±1.4</td>
<td>1.47±0.023***</td>
</tr>
<tr>
<td>Flunixin meg. (100 µg/ml)</td>
<td>8</td>
<td>23550</td>
<td>8000</td>
<td>41</td>
<td>33.97</td>
<td>5.1±1.2</td>
<td>1.39±0.018***</td>
</tr>
<tr>
<td>MMC (PC)² (0.2 µg/ml)</td>
<td>8</td>
<td>32000</td>
<td>8000</td>
<td>205</td>
<td>25</td>
<td>25.6±1.4***</td>
<td>1.25±0.009***</td>
</tr>
</tbody>
</table>

¹ (NC) Negative control. (PC) Positive control
² (CBPI) Cytokinesis-block proliferation index; by counting 1000 cells and calculated according to the formulation. CBPI = (MoN cell count + 2 x BN cell count + 3 x PN cell count) / total cell count (Fenech, 2000)

* p<0.05 *** p<0.001 (Compared with negative control group)
without metabolic activation at a dose of 100 µg/mL, which is similar to the highest dose in our study, and with metabolic activation at a concentration of 200 and 400 µg/mL (EMEA, 1999). In addition, in the present study, a significant increase (p<0.05) in MN frequencies at an even lower concentration of 50 µg/mL of flunixin meglumine could be due to the fact that the (MN) frequency in peripheral blood lymphocytes in conjunction with the CBMN assay is among the most popular and effective biomarkers used for evaluating the effect of genotoxic agents. (Fenech et al., 1999). Also, dose-dependent and reproducible positive results of flunixin meglumine were obtained in the mouse lymphoma forward mutation assay (EMEA, 1999).

In contrast to the occasional positive responses obtained by in vitro assays, in vivo data on the genotoxicity of flunixin meglumine are inconsistent with some positive and negative results (COM, 2003). Our negative results indicating a lack of chromosomal damage, measured as MN induction, agree with earlier studies performed in mammals in vivo. Flunixin meglumine was reported to give negative results in the mouse micronucleus test at dose levels of 40 and 80 mg/kg bw administrated intraperitoneally once a day for 2 days. Flunixin was negative in the same assay at dose levels of 100 and 150 mg/kg bw (EMEA, 1999). Meglumine, which is reported to be responsible for the genotoxicity of flunixin meglumine, was investigated in two separate micronucleus assays using BS1 and Alpk:ApfCD-1 mice. Positive results were obtained in BSI mice after intraperitoneal administration of 500 and 1000 mg/kg bw. However these results were not repeated in two bone marrow micronucleus assays in mice using an equivalent treatment regime. In contrast, it was reported that negative results were obtained in a separate in vivo micronucleus assay using intraperitoneal administration of two doses given 24 hours apart at up to 600 mg/kg bw of meglumine to CD1 mice. It was mentioned that the observed effect could be complicated by toxicity and there could be a considerable individual variation (COM, 2005). In order to avoid false-positive responses generated by nonphysiological conditions because of extremely high concentrations and toxicity, we used 100 mg/kg as the highest concentration which does not cause toxicity signs and mortality in mice.

It is difficult to account for the discordance between positive results in in vitro and negative results in in vivo/ in vitro mouse lymphocytes micronuclei. The possible explanation of differences between the results of the in vitro and in vivo/ in vitro assays of flunixin meglumine may be due to the alterations in its metabolic pathway that may be metabolised in vivo to a less genotoxic derivate or in vivo may be formed an adaptive response to flunixin meglumine.

With respect to cytotoxic effects of flunixin meglumine on lymphocyte cultures, as measured by CBPI, in comparison with the control value, a dose-dependent significant decrease in cell proliferation indicates a delay in the cell cycle progression which is an overt sign of cellular toxicity. Although, the reduction of CBPI was observed in the present study, no effect on the in vivo genotoxicity was observed even at the highest dose. This could be due to interaction of the compound with different cellular components resulting in cytotoxic and genotoxic effects.
In conclusion, our results indicate that flunixin meglumine has mutagenic potential in the cytochalasin B block micronucleus assay treated in vitro and has not genotoxic activity after in vivo administration evaluated with in vivo/in vitro micronucleus assay under test conditions. In addition, from CBPI data it is concluded that flunixin meglumine showed cytotoxic effects in cultured mouse lymphocytes both in vitro and in vivo/in vitro tests.

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ISPITIVANJE GENOTOKSIČNOSTI FLUNIKSIN MEGLUMINA UPOTREBOM IN VITRO I IN VIVO/IN VITRO MIKRONUKLEUSNOG TESTA

AYDIN SA i ÜSTÜNER KELES O

SADRŽAJ

Clij ovih ogleda je bio da se ispita genotoksični efekat fluniksin meglumina na perifere limfocite miša upotrebom in vitro i in vivo/in vitro blok-citokinetičkog mikronuleusnog testa (CBMN). Fluniksin meglumin je korišćen u koncentracijama od 25, 50 i 100 µg/ml za in vitro esej i 50, 75 i 100 mg/kg za in vivo/in vitro test. Miševi su tretirani intraperitonealno, dva puta u roku od 24 h i žrtvovani 6 h posle druge aplikacije. Uzorci krvi su uzimani punkcijom srca i kultivisani za in vivo/in vitro test. Nakon isteka 21 sata, od dodavanja testirane substancije za in vitro test, i posle inicijacije inkubacije za in vivo/in vitro test, citokinezija je blokirana dodava-
njem citohalazina-B. Čelijske kulture su analizirane dvadeset sati kasnije. U oba test sistema su korišćene negativne i pozitivne (mitomicin C - MMC) kontrole. Frekvenca pojavljivanja mikronukleusnih binuklearnih čelija (MNBN) je bila povećana nakon oba tretmana, ali su razlike između tretiranih i kontrolnih čelija bile značajne samo pri tretmanu in vitro. Ovo povećanje je bilo dozno-zavisno i značajan porast broja MNBN čelija (p<0,05 i p<0,001) je uočen pri koncentracijama od 50 i 100 μg/ml respektivno. Osim toga, oba tretmana su dovodila do smanjenja citokinetičkog blok proliferacijskog indeksa (CBPI) što ukazuje na citotoksičnost fluniksin meglumina.

Na osnovu ovih rezultata se može zaključiti, da fluniksin meglumin ispoljava genotoksične efekte prema limfocitima miša, tretiranim in vitro, ali nema mutagen nu aktivnost in vivo koja se može dokazati mikronukleusnim testom.