Evaluation of the Genotoxic Potential of the Selective COX-2 Inhibitor Enflicoxib in a Battery of *in vitro* and *in vivo* Genotoxicity Assays

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ABSTRACT

Aim Enflicoxib, a selective COX-2 inhibitor approved for the treatment of pain and inflammation associated with osteoarthritis in dogs (Daxocox® [Ecuphar/Animalcare Group]) was assessed for its genotoxic potential in a battery of *in vitro* and *in vivo* genotoxicity assays. These comprised a bacterial reverse mutation assay (Ames test), an *in vitro* human lymphocyte chromosome aberration assay and an *in vivo* mouse bone marrow micronucleus assay.

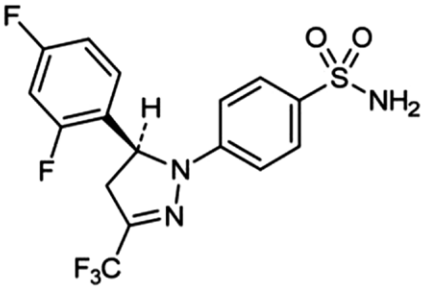
Methods Relevant vehicle and positive control cultures and animals were included in all assays. In the Ames test, enflicoxib was tested at concentrations of up to 5000 µg/plate. Signs of cytotoxicity were observed at the highest tested concentrations for several of the bacterial strains, both in absence and presence of S9. In human lymphocytes, enflicoxib was assessed for the induction of chromosomal aberrations when exposed at concentrations of up to 62.5 (3 hours) and 29.6 µg/mL (20 hours) in the absence of S9, and up to 66.7 µg/mL (3 hours) in presence of S9. Signs of cell toxicity, evidenced as a decrease in the mitotic index, were observed at these concentrations. In the mouse micronucleus assay, enflicoxib dose levels of up to 2000 mg/kg were administered (single dose) to male and female animals, and bone marrow samples were taken 24 and 48 hours (high-dose animals only) after administration.

Results Enflicoxib was shown to lack genotoxic activity in the conducted assays.

Conclusions The administration of enflicoxib as a therapeutic analgesic agent would not pose a genotoxic risk to animals or humans.

Introduction

Enflicoxib (also known by its research acronym E-6087) is a new pyrazoline derivative COX-2 inhibitor showing potent anti-inflammatory and analgesic activity when tested in experimental models of inflammation and pain in laboratory animals (▶ **Fig. 1**) [1] and in dog [2]. It has also shown to be effective in field clinical trials [3] with a broad safety margin when administered as recommended in dogs [4]. Enflicoxib has recently been approved for commercial veterinary use in Europe as Daxocox® tablets for dogs (Ecuphar NV/Animalcare) for the treatment of pain and inflammation associated with osteoarthritis in dogs, administered at a weekly dose of 4 mg/kg with an initial loading dose of 8 mg/kg [2].



▶ **Fig. 1** Chemical structure of enflicoxib.

Assessment for genotoxic potential is a mandatory step in the development process of a new pharmaceutical, as the exposure to agents with genotoxic activity is considered associated to the potential for induction of carcinogenesis and hereditary defects. It is therefore a common regulatory requirement to assess the genotoxic potential of both human and veterinary pharmaceuticals [5, 6]. To reduce the risk of genotoxic compounds not being properly detected, human and veterinary pharmaceuticals are required to be tested in a battery comprised of *in vitro* and *in vivo* genotoxicity assays [7, 8]. According to current veterinary legislation this generally comprises, assessment for induction of gene mutation in bacteria, *in vitro* chromosomal damage and/or gene mutation in mammalian cells, and an *in vivo* test assessing chromosomal damage in rodent haematopoietic cells.

We report here the results that were obtained when enflicoxib was assessed for genotoxic potential in a battery of GLP compliant *in vitro* and *in vivo* genotoxicity assays conducted according to internationally accepted standards. For this purpose, enflicoxib was tested in a bacterial reverse mutation assay (Ames test), an *in vitro* chromosome aberration assay in human lymphocytes and an *in vivo* mouse bone marrow micronucleus assay. The results obtained in these studies and their relevance for animal and human risk assessment is discussed in this manuscript.

Methods

Test substances

Enflicoxib (1-(4-aminosulfonylphenyl)-5-(2,4-difluorophenyl)-4,5-dihydro- 3-trifluoromethyl-1H-pyrazole, CAS 251442–94–1) was synthesized by Esteve Pharmaceuticals (Barcelona, Spain). Test item solutions were prepared by dissolving enflicoxib in dimethyl sulfoxide (DMSO) for the Ames test and the Chromosome Aberration assay. For the *in vivo* mouse micronucleus test enflicoxib was formulated as a suspension in 0.5 % methylcellulose. For *in vitro* studies, a stock solution was prepared at the maximum concentration required for the treatment and was diluted in appropriate sterile solvent to reach the required concentrations. Purity of enflicoxib was at least 99.5 %. Vehicles used for enflicoxib formulation were used as negative controls in the corresponding assays. Enflicoxib formulations were prepared immediately prior to testing. Positive control chemicals were obtained from the following suppliers: cyclophosphamide (CP, Sigma), ethylmethanesulfonate (EMS, Sigma), 2 Nitrofluorene (2NF, Aldrich), Sodium azide (NaN_3, Sigma), 4-Nitroquinoline oxide (4NQ, Sigma), 9-Aminoacridine (9AC, Sigma), and 2-Aminoanthracene (2-AA, Aldrich). For treatment, CP and NaN_3 were dissolved in water, EMS, 2NF, 4NQ, 9AC and 2-AA were dissolved in DMSO.

Metabolic activation system

For *in vitro* assays, commercial mammalian rat liver post-microsomal fraction (S9) was used as an exogenous metabolic activation system. S9 fractions prepared from male Sprague Dawley rat livers induced with Aroclor 1254 were obtained from Trinova Biochem GMBH (Gieben, Germany) and from ICN (USA) for the Ames test and chromosomal aberrations tests, respectively. S9 fractions were stored frozen at approximately − 80 °C, thawed just prior to use and supplemented with a NADPH-generating system (S9-mix). Final concentration of S9 fraction in the S-9 mix was 10 % for all *in vitro* assays.

Ames test

The method was carried out following the recommendations of Maron and Ames [9] and according to the corresponding OECD guideline [10]. Four *Salmonella typhimurium* histidine auxotrophic strains (TA98, TA100, TA1535 and TA1537) and an *Escherichia coli* tryptophan requiring strain (WP2 uvrA pKM101) were used. *Salmonella* strains TA98, TA100 and TA1535 were obtained from CECT (Spanish Collection of Type Cultures, Valencia, Spain), *Salmonella* strains TA1537 was obtained from Moltox (USA) and *Escherichia coli* strain from DSMZ (German Collection of Microorganisms and Cell Cultures, Germany). A frozen sample of each tester strain was thawed and grown in NB2 culture media until late-exponential culture phase. The potential for the solubility or cytotoxicity of enflicoxib to act as a dose limiting factor in the test system was assessed in a preliminary study with strain TA100 both in absence and presence of metabolic activation (results not shown).

The main study comprised two independent experiments performed on different days and using fresh cultures of each bacterial strain. Experiment 1 was performed by means of the direct plate incorporation method while Experiment 2 was carried out by means of the preincubation method. All experiments were performed both in absence and presence of metabolic activation, using triplicate plates for each tested substance and treatment condition. Tested concentrations and conditions are detailed in ▶ **Table 1**. DMSO was used as negative control. 2NF, AZ, 4NQ, 9AC and 2-AA were used as positive controls. Briefly, 0.1 mL of test item (or control solutions), 0.1 mL of bacterial culture and 0.5 mL of S9 mix (or phosphate buffer solution for treatments in absence of metabolic activation) were mixed and poured on to Vogel Bonner-E minimal agar plates after addition of 2 mL of top agar at 42 °C. In Experiment 2, this mix was incubated for 20 min at 37 °C, before addition of 2 mL molten agar at 42 °C.

Plates were inverted and incubated for 48–72 hours at 37 °C in the dark. Revertant colonies were counted by means of a Sorcerer image analyzer system (Perceptive Instruments) and the background lawn inspected for signs of toxicity or compound precipitation. From the individual plate counts the mean number of rever-

▶ **Table 1** Ames test.

Experiment 1										
	Mean number revertant colonies per plate ± SD[a]									
Treatment	**TA98**		**TA100**		**TA1535**		**TA1537**		***E.coli* WP2 *uvrA* (pKM101)**	
	−S9	**+S9**	**−S9**	**+S9**	**−S9**	**+S9**	**−S9**	**+S9**	**−S9**	**+S9**
Vehicle control	33.3 ± 10.50	35.0 ± 2.00	99.3 ± 9.29	92.7 ± 15.82	16.0 ± 6.00	11.7 ± 2.08	9.0 ± 3.61	15.3 ± 2.31	97.3 ± 3.51	114.0 ± 5.57
Concentration (µg/plate)										
21	31.7 ± 4.62	34.7 ± 7.51	109.7 ± 4.04	92.3 ± 25.11	14.3 ± 3.51	13.0 ± 5.57	13.0 ± 5.57	10.3 ± 3.51	106.3 ± 10.97	130.3 ± 6.66
62	35.7 ± 7.37	27.3 ± 2.31	99.7 ± 3.79	96.3 ± 6.81	10.3 ± 2.08	9.7 ± 4.04	9.7 ± 3.79	14.3 ± 2.08	98.3 ± 6.66	122.3 ± 4.16
185	27.3 ± 2.52	42.0 ± 7.00	96.3 ± 12.58	97.7 ± 12.90	11.7 ± 7.02	9.7 ± 4.62	8.0 ± 4.00	8.0 ± 0.00	82.0 ± 7.55	114.7 ± 1.53
556	30.0 ± 5.29	38.0 ± 8.19	110.0 ± 25.24	103.0 ± 6.24	7.3 ± 3.06	17.3 ± 2.31	8.0 ± 1.00	8.7 ± 3.21	86.0 ± 4.58	135.3 ± 7.51
1667	31.0 ± 7.81	15.0 ± 5.29	90.3 ± 4.73	88.0 ± 5.57	8.0 ± 1.73	4.3 ± 1.53	8.0 ± 1.73	1.3 ± 0.58	88.3 ± 8.62	62.7 ± 5.69
5000	22.3 ± 7.64	12.0 ± 5.57	98.0 ± 10.15	78.7 ± 8.74	12.3 ± 1.15	1.7 ± 0.58	9.3 ± 3.21	1.3 ± 0.58	78.7 ± 9.07	61.0 ± 2.65
Positive Control[b]	122 ± 11.79	236 ± 40.50	583 ± 36.47	569 ± 25.58	284 ± 3.51	107 ± 15.72	258 ± 61.78	147 ± 8.33	874 ± 53.20	471 ± 21.73
Experiment 2										
	Mean number revertant colonies per plate ± SD[a]									
Treatment	**TA98**		**TA100**		**TA1535**		**TA1537**		**E.coli WP2 uvrA (pKM101)**	
	−S9	**+S9**	**−S9**	**+S9**	**−S9**	**+S9**	**−S9**	**+S9**	**−S9**	**+S9**
Vehicle control	26.0 ± 1.00	43.0 ± 6.56	97.0 ± 9.85	112.3 ± 5.77	15.3 ± 1.15	16.7 ± 4.62	10.7 ± 3.51	19.3 ± 3.06	115.3 ± 13.58	179.0 ± 17.09
Concentration (µg/plate)										
21	32.0 ± 2.65	29.7 ± 7.23	109.7 ± 20.55	105.0 ± 3.00	15.7 ± 0.58	10.7 ± 4.04	7.7 ± 3.51	13.0 ± 1.73	107.3 ± 18.34	147.0 ± 11.36
62	25.3 ± 9.02	38.0 ± 3.00	113.3 ± 4.73	111.0 ± 11.53	13.0 ± 4.36	17.3 ± 8.74	9.7 ± 1.53	13.3 ± 8.33	114.0 ± 4.36	120.7 ± 3.51
185	42.3 ± 3.21	38.3 ± 5.77	114.7 ± 12.50	90.3 ± 5.03	17.3 ± 6.81	15.3 ± 0.58	9.7 ± 5.51	11.7 ± 2.08	116.3 ± 8.02	122.0 ± 12.17
556	21.7 ± 3.21	33.7 ± 9.07	116.7 ± 14.43	89.7 ± 9.87	17.3 ± 7.09	12.7 ± 4.62	9.3 ± 2.08	12.7 ± 3.06	109.7 ± 10.02	132.7 ± 10.02
1667	21.3 ± 4.04	15.3 ± 4.51	109.0 ± 4.36	78.7 ± 10.79	11.0 ± 1.73	9.3 ± 2.52	8.3 ± 6.66	3.3 ± 0.58	74.3 ± 8.39	76.7 ± 1.53
5000	30.7 ± 2.08	17.3 ± 4.93	123.0 ± 22.72	78.3 ± 10.69	9.3 ± 1.15	7.7 ± 1.53	5.0 ± 1.73	3.7 ± 1.15	65.0 ± 5.57	59.7 ± 2.08
Positive Control[b]	172 ± 44.11	251 ± 39.23	747 ± 160.59	696 ± 85.17	553 ± 196.59	129 ± 20.66	977 ± 206.41	161 ± 27.32	1580 ± 157.72	417 ± 45.94

(a) Mean of 3 replicas for all tested conditions. (b) In absence of S9, TA98: 2-NF (2 µg/plate); TA100: AZ (1 µg/plate); TA1535: AZ (0.25 µg/plate); TA1537: 9-AC (50 µg/plate); E. coli WP2 uvrA (pKM101): NQ (0.125 µg/plate). In presence of S9, 2-AA: TA98, TA100, TA1535 (1 µg/plate); TA1537 (2 µg/plate); E. coli WP2 uvrA (pKM101) (10 µg/plate).

tants and standard deviation (SD) for each test item, concentration and experimental condition were determined. The assay was considered positive for mutagenic activity when there was a greater than 2-fold increase in the mean number of revertants versus the mean number of spontaneous revertants obtained in the vehicle control in any of the test strains, and a dose-response effect was observed.

Human lymphocyte chromosome aberration assay

The test was conducted following the method described by Dean and Danford [11] and following the recommendations of the corresponding OECD guideline [12]. Two independent experiments were performed. Whole blood heparinized (50 IU/mL) samples were obtained from healthy non-smoking adults (male and a female subject for Experiments 1 and 2, respectively). Cultures were established by placing 0.5 mL of whole blood into 9.5 ml RPMI 1640 Dutch-modified medium supplemented with 10 % foetal calf serum, 50 µg/mL streptomycin, 50 IU/mL penicillin, 5 µg/mL phytohaemaglutinin and 1 IU/mL heparin. Cultures were incubated for approximately 48 hours at 37 °C in a 5 % of CO_2 environment and shaken gently during culture period, prior to the start of treatment. For each treatment condition, duplicate cultures for each enflicoxib concentration and positive controls and quadruplicate cultures for vehicle control were set up. Tested concentrations and conditions are detailed in ▶ **Table 2**. Vehicle-control cultures were treated with DMSO, and positive control cultures with EMS and CP in the absence and presence of S9, respectively. Enflicoxib and controls were administered at 1 % (v/v) into the culture media. In Experiment 1, cultures were treated for 3 hours in absence and presence of S9 followed by 17 hours recovery. In Experiment 2, cultures were treated for 3 hours in presence of S9, followed by a 17 hours recovery period, while cultures treated in absence of S9 were exposed continuously for 20 hours to enflicoxib.

The concentration of S9-mix in the treatment medium was 10 % (final concentration of S9, 1 %). After the 3 hours treatment period in presence or absence of S9 mix, cells were washed twice with sterile saline and resuspended in fresh medium for the completion of the recovery phase. Colcemid (Serva) at a final concentration of 0.4 µg/mL was added 3 hours prior to harvest. Cells were collected by centrifugation (10 minutes 600 g), resuspended in potassium chloride hypotonic solution (0.075 M) for 20 minutes and fixed in ice-cold methanol/glacial acetic acid solution (3:1, v/v). Several drops of the cell suspension were spread on microscope slides. The slides were air-dried and stained with Giemsa 5 % in Sorensen buffer at pH 6.8 for 5 minutes. To assess cytotoxic/cytostatic effects, the mitotic index (MI) for each culture was established by examining the presence of metaphases in 1000 cell nuclei. After MI assessment, three test substance concentrations (with and without S9) were selected for cytogenetic analysis. For vehicle and positive controls and each test substance concentration, 200 well-spread metaphase figures were analysed for the presence of structural chromosomal aberrations (100/culture). The classification of structural aberrations was carried following the guidance of the International System for Cytogenetic Nomenclature [13]. The presence of polyploid, endoreduplicated and hyperdiploid cells was also recorded. For each treatment condition the proportion of cells with structural aberrations excluding gaps was compared with that of the corresponding vehicle control using Fisher's exact test [14]. The assay was considered positive when there was a statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) at any concentration that exceeded the historical vehicle control range, and the effect was reproducible.

Mouse bone marrow micronucleus test

The micronucleus test was conducted following the method of Schmid [15] and following the recommendations of the corresponding OECD guideline [16]. CD-1 mice of the Crl:CD-1(ICR)BR strain were obtained from Charles River Laboratories (UK). All procedures involving animals and their care were conducted in strict conformity with the European Community and local Guide for the Care and Use of Laboratory Animals [17, 18], and the protocol was reviewed and approved by the testing facility ethical committee. The animals underwent a minimum 5-day acclimatization period to the environmental conditions of the room before the beginning of the assay. Animals were randomly allocated in groups of up to 5 animals and housed in Makrolon® cages with sawdust bedding. Animals were housed in rooms with temperature and relative humidity set at target values of 22 °C and 30–70 %, respectively; lighting was controlled to give a 12-h light-dark cycle; air conditioning guaranteed a minimum of 18 volume air changes per hour. They had access *ad libitum* to water and commercial rodent diet (Panlab SL, Spain).

On the day of dosing animals were approximately 8–9 weeks of age and in a weight range of 29–39 g for males and 23–28 g for females. Animals were dosed by oral route (gavage) at a dose volume of 10 mL/kg. The animals were weighed immediately before administration and received a weight-matched volume of vehicle, formulated test substance, or positive control. Based on a preliminary study, enflicoxib doses of 500, 1000 and 2000 mg/kg were selected. Groups of 5 male and 5 female animals each received either vehicle (0.52 % Methylcellulose), CP (50 mg/kg) or enflicoxib at each of the stated doses and were sacrificed by cervical dislocation 24 hours after administration. Two further groups receiving the vehicle or the enflicoxib high dose were sacrificed 48 hours after administration (▶ **Table 3**). Immediately after euthanasia, the femurs were dissected, bone marrow extracted, placed on a slide containing a drop of foetal calf serum (Sigma, UK) and a direct smear was obtained (two slides per animal). Smears were fixed with methanol and stained with 5 % Giemsa. Slides were coded and examined under light microscopy (Olympus BH2). For each animal, the relative proportion of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) was determined by counting a total of 1000 erythrocytes. The number of micronucleated polychromatic erythrocytes (MNPCE) among 2000 PCE was determined. A total of 1000 normochromatic erythrocytes (NCE) was also observed and the number with micronuclei was also recorded. The number of MNPCE in each treated group (males and females separately and combined) were compared with the corresponding vehicle group by means of the Dunnett's test [19] after rank transformation of data. The result was considered positive when there was a statistically significant increase ($p < 0.05$) in the frequency of MNPCE, at least at one dose, that exceeded the historical vehicle control range.

▶ **Table 2** Chromosome aberration assay.

Experiment 1												
Treatment	**Concentration (µg/mL)**	**S9**	**Exposure-recovery (h)**	**Structural aberrations**								
				Cells scored	**Mit. Index**	**Gaps**	**Chromosome**		**Chromatid**		**Cells with aberrations**	
					(%)		**Deletion**	**Exchange**	**Deletion**	**Exchange**	**Incl. Gaps**	**Excl. Gaps**
Vehicle	–	–	3–17	400	100[A]	1	0	0	4	0	5	4
Enflicoxib	15.6	–	3–17	200	74	1	0	0	1	0	2	1
	31.2	–	3–17	200	62	3	1	0	2	0	6	3
	62.5	–	3–17	200	30	3	1	0	2	0	6	3
EMS	750	–	3–17	200	41	5	2	0	34	12	44***	41***
Vehicle	–	+	3–17	400	100[A]	2	0	0	3	0	5	3
Enflicoxib	15.6	+	3–17	200	81	0	0	0	3	0	3	3
	31.2	+	3–17	200	69	1	0	0	2	0	3	2
	62.5	+	3–17	200	56	2	0	0	2	0	4	2
CP	4	+	3–17	200	38	11	1	0	23	1	31***	24***
Experiment 2												
Treatment	**Concentration (µg/mL)**	**S9**	**Exposure-recovery (h)**	**Structural aberrations**								
				Cells scored	**Mit. Index**	**Gaps**	**Chromosome**		**Chromatid**		**Cells with aberrations**	
					(%)		**Deletion**	**Exchange**	**Deletion**	**Exchange**	**Incl. Gaps**	**Excl. Gaps**
Vehicle	–	–	20–0	400	100[B]	4	3	0	20	0	24	21
Enflicoxib	13.2	–	20–0	200	74	5	0	0	12	0	15	10
	19.8	–	20–0	200	67	2	0	0	10	0	11	10
	29.6	–	20–0	200	54	4	0	0	2	1	7	3
EMS	250	–	20–0	200	49	12	1	1	44	0	47***	37***
Vehicle	–	+	3–17	400	100[B]	5	1	0	14	0	18	13
Enflicoxib	29.6	+	3–17	200	79	2	0	0	6	0	8	6
	44.4	+	3–17	200	77	2	0	4	5	0	8	6
	66.7	+	3–17	200	61	1	3	0	6	0	10	9
CP	4	+	3–17	200	26	9	2	0	45	4	48***	42***

***$p < 0.001$; Fisher's exact test; CP: Cyclophosphamide; EMS: Ethyl methanesulfonate; A: Absolute mitotic index values were 72.8‰ and 70‰ in absence and presence of S9, respectively; B: Absolute mitotic index values were 69.8‰ and 69.3‰ in absence and presence of S9, respectively.

▶ **Table 3** Single dose mouse micronucleus test.

Sampling time	Treatment	Dose (mg/kg)	N	PCE/NCE (mean ± SD)	MNPCE/1000 PCEs (mean ± SD)	MNNCE/1000NCEs (mean ± SD)
24 h.	Vehicle	–	10	1,31 ± 0,44	1,3 ± 0,4	1,1 ± 1,1
	Enflicoxib	500	10	1,49 ± 0,32	1,9 ± 0,8	0,7 ± 0,5
		1000	10	1,92 ± 0,61	1,8 ± 1,2	1,1 ± 0,9
		2000	10	1,53 ± 0,76	1,8 ± 0,9	1,2 ± 0,6
	CP	50	10	0,85 ± 0.29	31,1*** ± 14.3	1,8 ± 1,5
48 h.	Vehicle	–	10	1,53 ± 0,52	1,6 ± 0,6	1,4 ± 1,3
	Enflicoxib	2000	10	1,57 ± 0,35	2,2 ± 1,5	0,6 ± 0,7

***p < 0.001 Wilcoxon's sum of rank test; CP: Cyclophosphamide.

Results

Ames test

In the preliminary study with strain TA100, compound precipitation was observed at the two highest tested concentrations of 1667 and 5000 µg/plate. However, as these concentrations were not associated with marked toxicity or interfered with the scoring of revertants, a concentration range of up to 5000 µg/plate was tested in the main study. In the two independent conducted assays of the main study, enflicoxib was tested at concentrations of up to 5000 µg/plate both in presence and absence of a rat liver metabolic activation system (S9-mix). Under all tested conditions, no increase in the number of revertants compared to the vehicle control values were observed. Results are shown in ▶ **Table 1**. In Experiment 1, evidence of cell cytotoxicity, as shown by a reduction (exceeding 50 %) in the number of colonies compared to the vehicle control, were observed in strains TA98, TA1535 and TA1537 at concentrations ≥ 1667 µg/plate, in the presence of metabolic activation. Precipitation of enflicoxib in the culture plates was observed in the whole battery of strains at concentrations ≥ 1667 µg/plate, in the presence and in the absence of metabolic activation. Likewise, test item precipitation was also observed at the 556 µg/plate concentration, in the absence of metabolic activation, in strains TA98, TA1535 and *E. coli* WP2 *uvrA* pKM101.

In Experiment 2, a reduction (exceeding 50 %) in the number of colonies compared to the vehicle control was observed in strains TA 98, TA1537 and *E. coli* WP2 *uvrA* pKM101 at concentrations ≥ 1667 µg/plate, and in strain TA1535 at the 5000 µg/plate concentration, in the presence of metabolic activation. In the absence of metabolic activity, signs of cytotoxicity were observed in strain TA1537 at the 5000 µg/plate concentration. Test item precipitation was observed in the whole battery of strains at concentrations ≥ 1667 µg/plate, in the presence and in the absence of metabolic activation. Likewise, test item precipitation was also observed in strain TA98 at the 556 µg/plate concentration, in the absence of metabolic activation. The positive controls employed in the assays produced clear increases in the number of revertants in comparison to the vehicle control. It was concluded that enflicoxib was not mutagenic in this bacterial system.

Human lymphocyte chromosome aberration assay

In Experiment 1, human peripheral blood lymphocytes were exposed to enflicoxib concentrations of 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg/mL, both in the absence and presence of metabolic activation, for 3 hours. Concentrations ≥ 125 µg/mL proved to be extremely toxic, both in the absence and presence of S-9 mix, with almost complete absence of viable cells in the treated cultures. The next lower concentration of 62.5 µg/mL, showed a decrease in the mitotic index down to 30 and 56 % of the matching vehicle control value in the absence and presence of S-9 mix, respectively, and was selected as the high concentration for metaphase analysis. The 31.2 and 15.6 µg/mL concentrations were selected as intermediate and low concentrations, respectively. Results of the slide examination are shown in ▶ **Table 2**.

In Experiment 2, human peripheral blood lymphocytes were exposed to enflicoxib concentrations of 5.8, 8.8, 13.2, 19.8, 29.6, 44.4 and 66.7 µg/mL in the absence of metabolic activation (20 hours) and at concentrations of 13.2, 19.8, 29.6, 44.4, 66.7 and 100 µg/mL in the presence of metabolic activation (3 hours). In the absence of metabolic activation, concentrations ≥ 44.4 µg/mL proved to be extremely toxic with a null or very small number of metaphases present. The next lower concentration of 29.6 µg/mL, that showed a decrease in the mitotic index down to 54 % of the vehicle control value, was selected as the highest concentration for metaphase analysis. The 19.8 and 13.2 µg/mL concentrations were selected as intermediate and low concentrations, respectively. In the presence of S-9 mix, the highest tested concentration (100.0 µg/mL) proved to be extremely toxic, with total absence of cell material in the obtained preparations. The next lower concentration of 66.7 µg/mL, showed a decrease in the mitotic index down to 61 % of the vehicle control value and was selected as the high concentration for metaphase analysis. The concentrations of 44.4 and 29.6 µg/mL were selected as intermediate and lowest concentrations, respectively.

Both in Experiment 1 and 2, no increase in the number of structural or numerical chromosomal aberrations was observed in cultures treated with enflicoxib under any of the test conditions. The positive controls EMS and CP produced marked and statistically significant increases ($p < 0.001$) in the number of chromosomal aberrations, in absence and presence of metabolic activation, respectively, confirming the sensitivity of the test system. It was concluded that enflicoxib did not show genotoxic activity in this *in vitro* experimental assay.

Mouse bone marrow micronucleus test

No mortality or clinical signs were recorded among animals receiving enflicoxib in the micronucleus test. Results of bone marrow examination are shown in ▶ **Table 3**. There was no effect of enflicoxib administration on PCE/NCE ratio at any dose level or sampling time. The mean frequency values of MNPCEs in the different enflicoxib treated groups were similar to those of their corresponding vehicle treated groups. The positive control CP induced, at the 24 hours sampling time, a slight decrease in the PCE/NCE ratio and a marked and statistically significant increase ($p < 0.001$) in MNPCE frequency, confirming the sensitivity of the test system. It was concluded that enflicoxib showed no potential for genotoxic activity in this *in vivo* assay.

Discussion

The selective COX-2 inhibitor enflicoxib was assessed for genotoxic potential by means of a battery of genotoxicity assays, comprising a bacterial reverse mutation assay (Ames test), a human lymphocyte chromosome aberration assay, and a mouse bone marrow micronucleus test. Testing for genotoxicity aims at identifying the potential to cause DNA damage, for this being considered essential for the induction of carcinogenesis and hereditary defects. Compounds that are positive for genotoxicity are considered being potentially carcinogens and/or mutagens in animals and humans. Though the regulatory requirement for assessing the genotoxic potential of veterinary drugs in a strict sense is formulated in the context of the safety of "veterinary drug residues in human food" [8] or the "safety for the user" [20], thereby placing its main focus on human risk, it will undoubtedly also apply to the risk imposed to the veterinary target animal species, this being particularly relevant for pet-animal species.

Within the so called "standard battery" of genotoxicity assays, the *in vitro* studies are generally considered to be the most sensitive for detecting genotoxic carcinogens, a contributing factor being that the exposure of the target cells is less problematic compared with *in vivo* assays. To this respect, in the *in vitro* assays conducted during our investigations, i. e. Ames test and human lymphocyte chromosome aberration assay, enflicoxib was clearly shown to lack genotoxic activity even when tested at concentrations that were associated to sings of cytotoxicity. In the Ames test, this was manifested as a reduction in the number of revertants at concentrations of ≥ 1667 µg/plate. In the chromosome aberration assay, there was an absence of viable cells at concentrations of ≥ 125 and ≥ 44.4 µg/mL in the absence of metabolic activation (3 and 20 h exposure, respectively), and at ≥ 100 µg/mL in the presence of metabolic activation (3 h exposure). The potential for enflicoxib to produce cytotoxic effects in bacterial and/or mammalian cells at concentrations that are within the range of the regulatory required test concentrations for assessing genotoxicity, is shared by other structurally related drugs pertaining to the same pharmacological class (COX-2 inhibitors), such as celecoxib (Celebrex®) and valdecoxib (Bextra®) [21, 22]. Celecoxib was reported to be toxic in the different tested bacterial strains (TA97a, 98, TA100, TA1535, TA1538) at concentrations of ≥ 500 µg/plate, while no such effect was reported for valdecoxib. When assessed for the *in vitro* induction of chromosomal aberrations in mammalian cells (CHO), marked cytotoxicity was reported at concentrations of ≥ 80 µg/mL for celecoxib, and of ≥ 117 µg/mL or ≥ 75 µg/mL (4 and 24 h exposure, respectively) for valdecoxib. As for enflicoxib, both compounds were reported to lack clastogenic activity. However, for celecoxib an increased incidence of cell endoreduplication was reported when treated in presence of metabolic activation at ≥ 30 µg/mL [21]. Presence of endoreduplication is not considered to necessarily indicate potential for spindle damage and aneuploidy, and there is evidence that endoreduplication may indicate cell cycle perturbation. [23]. On the contrary, the COX-2 inhibitor parecoxib was reported to produce chromosomal aberrations in CHO cells after short term exposure (4 hours) both in the absence and the presence of S9, although this genotoxic effect was not reproduced when tested *in vivo* for genotoxicity [24].

In vivo assays are considered a pivotal component in the overall assessment of genotoxic potential, as they can take into account the biological processes of absorption, distribution, metabolism and excretion, which are not accounted for by *in vitro* assays. As such, they are considered to mimic closer the human situation and their results are given a great regulatory weight. Although failure of *in vitro* assays to detect relevant *in vivo* genotoxins is a rare occurrence, compounds such as procarbazine, urethane and benzene are examples of *in vivo* genotoxins which have proved difficult to be consistently detected in common regulatory *in vitro* assays [25, 26]. When tested for *in vivo* genotoxic potential, enflicoxib was shown to lack genotoxic activity, either clastogenic or aneugenic, as evidenced by the lack of induction of MN in mouse bone marrow erythrocytes, after single dose oral administration of up to 2000 mg/kg dosages. The peak (Cmax) systemic exposure to enflicoxib achieved in mice after oral administration of a 2000 mg/kg dose, as determined in an independent pharmacokinetic study (unpublished results), was approximately 15-times the highest exposure achieved in the target animal species under therapeutic conditions [27].

Conclusions

Enflicoxib was shown to lack genotoxic potential when thoroughly assessed in a battery of genotoxicity assays which comprised, a bacterial reverse mutation assay (Ames test), a human lymphocyte chromosome aberration assay and a mouse bone marrow micronucleus assay. From the results obtained it is concluded that administration of enflicoxib would not pose a genotoxic risk to humans or target animal species.

Conflicts of Interest

JH and MS are employees of Ecuphar Veterinaria SLU (Animalcare group), who sponsored the study. AG declares no conflict of interest.

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