

Assessment of the Genotoxic Potential of Cizolirtine a Substance-P and Calcitonin Gene-Related Peptide Release Modulator

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ABSTRACT

The analysis of the genotoxic potential of cizolirtine, a compound being developed as a drug for analgesia and for urinary incontinence, was carried out using a battery of *in vitro* and *in vivo* assays as recommended in the guidelines for medicinal products. Negative results were obtained in an Ames test (up to 5000 µg/plate), in a Mouse Lymphoma assay (up to 2000 µg/ml) and in a single dose mouse bone marrow micronucleus assay (up to 300 mg/kg). In a human lymphocyte chromosome aberration assay, a slight statistical increase in the frequency of cells with chromosome aberrations including gaps was reported for the concentrations of 200 and 1600 µg/ml at the 24-h sampling time. This minor increase in chromosome aberrations was considered of questionable biological relevance since it was moderate, was within the laboratory historical control values, did not show a dose-dependent effect and was not observed at similar concentrations in a repeat assay. Taking into considerations the results obtained in the different *in vitro* and *in vivo* assays and a weight-of-evidence analysis, it suggests that cizolirtine would not pose a genotoxic risk when administered to humans.

Introduction

Cizolirtine [(±)-5- α -[2-(dimethylamino)ethoxy]benzyl]-1-methyl-1H-pyrazol citrate (► **Fig. 1**) is a drug with a potent analgesic profile, that has shown its antinociceptive properties in several rodent models of acute pain. More precisely, cizolirtine is able to reduce in a dose-dependent manner the pain caused by chemical (i.p. injection of phenylbenzoquinone or acetic acid), thermal (tail-flick), or mechanical stimuli (tail-pinch). In a similar way, it prevents pain-associated behaviors induced by intradermal injection of either formalin or capsaicin and is able to decrease thermal hyperalgesia evoked by subplantar injection of carrageenan [1, 2]. Such results concur with data obtained in human volunteers, where cizolirtine decreased pain produced by thermal and electrical stimuli in a

dose-related manner [3], and was found to mitigate the pain caused by third molar extraction [4]. Cizolirtine also showed its antinociceptive activity in animal models of chronic painful neuropathy, alleviating allodynia against cold and mechanical stimulations in rats that suffered sciatic nerve constriction [3, 5]. In the same way, cizolirtine demonstrated to induce a dose-dependent antihyperalgesia in the streptozotocin (STZ)-induced diabetic neuropathy model [6]. These results are in line with the ones obtained in clinical trials, where significant reduction in pain effects of this drug in patients under conditions of posttraumatic/postherpetic neuropathy [7], cancer pain, and diabetic neuropathy [3] has been shown.

Due to *in vivo* studies showing that cizolirtine significantly reduced the spinal release of substance P and calcitonin gene-related peptide (CGRP) in both healthy and diabetic hyperalgesic rats, it has been attributed to this drug a role as a modulator of the release of these two neuropeptides involved in the transmission of nociceptive signals from the periphery to the central nervous system [6, 8]. This modulation effect on substance-P and CGRP is likely linked to the increase of noradrenaline and serotonin levels at the extracellular space. The action of noradrenaline and serotonin on the noradrenergic pathway induces a decrease in the release of substance P and CGRP at supraspinal level in the primary fibers [2, 6, 8]. These effects are the basis for its beneficial potential in the inflammation process of the urinary bladder, where visceral regulation of the motility would be facilitated by means of tachykinin control and therefore could be potentially favourable in certain forms of urinary incontinence [9].

Overactive bladder is a syndrome with high prevalence in both, women and men causing a negative impact on their quality of life [10, 11]. Nowadays, a remarkable number of antimuscarinic drugs are marketed them being the first-line drug therapy for overactive bladder [12]. The efficacy of cizolirtine on overactive bladder has been shown at preclinical level in animal models. More precisely, in a model of increased intraluminal pressure in rats, cizolirtine is able to reduce the vesical contractions width without modifying their rhythm. Moreover, cizolirtine produces a clear effect on urodynamic function in a rat model of isovolumetric rhythmic bladder contractions. Yet, cizolirtine is active in protecting from the effects of the intrabladder infusion of acetic acid in rats, thus regularizing the micturition frequency and volume and the area under the curve of intravesical pressure related to time (Saenz de Tejada, 2005 unpublished results). In a phase II proof of concept clinical trial cizolirtine improved significantly bladder diary variables versus placebo: voidings number per 24 h were reduced, mean voided volume per voluntary micturition was increased and the number of patients achieving less than eight voidings per 24 h and/or complete dryness was also increased. Altogether, cizolirtine shows a promising

profile in the treatment of overactive bladder with urinary incontinence [13, 14].

In this publication we report the results of the battery of genotoxicity studies as recommended by regulatory authorities as part of the preclinical safety assessment program for the development of cizolirtine as a drug to alleviate the effects of the two above mentioned disease conditions.

Material and Methods

Test substance

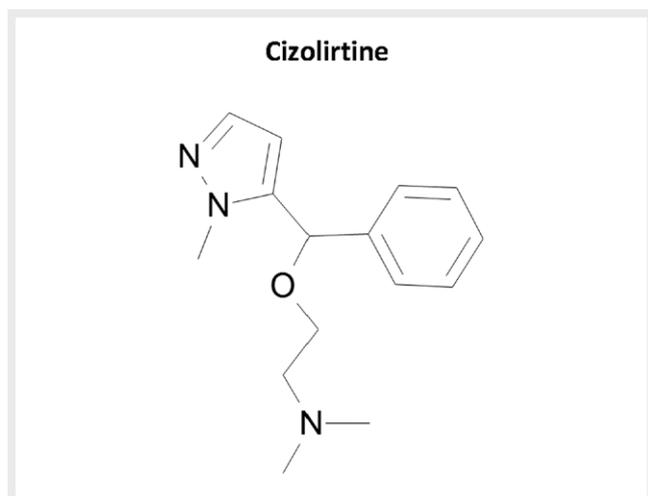
Cizolirtine bulk powder was synthesized by ESTEVE (Barcelona, Spain) (► Fig. 1). Cizolirtine was administered as a citrate salt in all studies with purity always above 95.5%. Test article solutions were prepared by dissolving cizolirtine in reverse-osmosis water immediately prior to the Ames Test and Mouse Lymphoma (ML) assays. For the Chromosome Aberration (CA) assay, the compound was dissolved in dimethyl sulfoxide (DMSO). Likewise, the solvent used in Mouse micronuclei assay was 0.9% saline solution. 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. The test material formulations were prepared as near as possible to the time of dosing. The vehicle (either, 0.9% saline solution, DMSO or reverse-osmosis water) was used as negative control for all studies. Positive control chemicals were obtained from the following manufacturers: cyclophosphamide (CP, Sigma), ethyl methanesulfonate (EMS, Sigma), methyl methanesulfonate (MMS, Sigma), 3-methylcholanthrene (3-MC, Sigma), 4-nitroquinoline 1-oxide (NQO, Sigma), 2-nitrofluorene (2NF, Aldrich), sodium azide (NaN_3 , Sigma), 9-aminoacridine (AAC, Sigma), 2-aminoanthracene (AAN, Aldrich). NaN_3 and CP were dissolved in water, while the remaining positive control chemicals were dissolved in DMSO.

Metabolic activation system

Mammalian liver post-mitochondrial fraction (S9) was used for metabolic activation in all *in vitro* experiments. S9 fractions were generated from the livers of male Sprague-Dawley (Ames Test and CA assay) or Fischer 344 (ML assay) rats induced with Aroclor 1254. S9 post-mitochondrial fraction were purchased from Organon Teknika (CA), ICN Cappel (Ames Test) or Harlan UK (ML). Fractions were stored frozen at approximately -80°C . Just prior to use, fractions were thawed and supplemented with a NADPH-generating system. Final concentration of the S9 fraction in the treatment medium for all *in vitro* assays was 10%.

Animal housing

Animals that intervened in the *in vivo* micronuclei assay were housed in rooms set at target values of 22°C of temperature and 50% of relative humidity. On the other hand, lighting was controlled to give a 12 h light-dark cycle while the air conditions were guaranteed setting a minimum of 15 volume changes of air per hour. All procedures involving animals and their care were conducted in strict conformity with the European Community Guide for the Care and Use of Laboratory Animals [15].



► Fig. 1 Cizolirtine's chemical structure.

Ames test

The method used was the described previously [16] following the recommendations of the corresponding OECD guideline [17]. *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 (histidine auxotrophic strains) and *Escherichia coli* (WP2 *uvrA* pKM101) (auxotrophic for tryptophan) were conforming the panel of the study. The *Salmonella* strains were obtained from CECT (Spanish Type Culture Collection, Spain) while the *E. coli* strain was obtained from NCIBM Ltd (National Collection of Industrial Food and Marine Bacteria, UK). A frozen sample of each tester strain was thawed and grown in NB2 culture media until late-exponential culture phase.

Two independent experiments were conducted, both in the presence and in the absence of metabolic activation. Each concentration of test substance, or either positive or negative control, was tested in triplicate plates. Test concentrations and conditions are detailed in ► **Table 1**. 2NF, NaN₃, NQO, AAC, and AAN were used as positive controls, and sterile reverse-osmosis water as vehicle control. Experiment 1 followed the plate-incorporation assay procedure whereas for Experiment 2, the preincubation method was used. Briefly, 0.1 ml of test substance (or control solution), 0.1 ml of bacterial culture and 0.5 ml of 10 % S9 mix (phosphate buffer solution for treatment without 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 the case of Experiment 2, this mix was incubated for 20 min at 37 °C, before addition of 2 ml molten agar at 42 °C.

Plates were incubated at 37 °C in the dark for 48–72 h. After this period of time, revertant colonies were counted. In order to assess toxicity signs the background lawn was inspected and the potential decrease in the number of colonies compared to the negative control was also considered. From the individual plate counts the mean and standard deviation for each treatment were determined. The one-way of the variance [18] was used to compare counts of each test substance concentration with its corresponding vehicle control. The Duncan test was used in case significance was found. Statistical significance was not the only criteria to determine a positive response as biological relevance was also a criterion taken into account. The compound was considered to be mutagenic when there was a reproducible increase of revertants at one or more concentrations per plate in at least one strain with or without metabolic activation system respect to the negative control.

Human lymphocyte chromosome aberration assay

Chromosome aberration assay was conducted following the method described by Dean and Danford [19] following the 473 OECD guideline [20]. Two independent experiments were performed both in the presence and in the absence of activation. Whole blood samples were obtained from two healthy adult subjects (female and male for Experiments 1 and 2, respectively) in heparinized tubes. Cultures were established by placing 0.4 ml of whole blood into 9 ml RPMI 1640 Dutch-modified medium supplemented with 10 % foetal calf serum, 50 µg/ml gentamycin and 5 µg/ml phytohaemagglutinin.

Cultures were incubated for approximately 48 h at 37 °C in a 5 % of CO₂ environment and rocked intermittently, prior to start of treatment. Both for treatment with and without S9, quadruplicate cultures for vehicle and duplicate cultures for each test substance concentration and the positive controls were set up. Test concen-

trations and conditions are detailed in ► **Table 2**. Vehicle-control cultures were treated with DMSO and positive control cultures with EMS or CP in the absence and presence of S9, respectively. In experiments 1 and 2, cultures were treated for 24 h in the absence of S9, and for 3 h in the presence of S9, followed by 21 h recovery. In Experiment 2 an additional 48 h treatment in the absence of S9, and for 3 h treatment in the presence of S9 followed by 45 h recovery was also performed. After treatment in the presence of S9, cells were washed twice with sterile saline and resuspended in fresh medium for completion of the recovery phase. Colcemid at a final concentration of 0.4 µg/ml was added 3 h prior to harvest. In Experiment 1, osmolality and pH measurements were performed in post-treatment media both without and with S9. Cells were collected by centrifugation (10 min 600 g), resuspended in hypotonic solution (0.075 M KCl) for 15 min, and fixed in ice-cold methanol/glacial acetic acid solution (3:1, v/v). A few 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 min. After mitotic index (MI) assessment, three test substance concentrations (with and without S9) were selected for cytogenetic analysis. For vehicle control and each test substance concentration, 200 well-spread metaphase figures were analyzed for the presence of structural chromosomal aberrations. In the case of the positive controls, 50 metaphase figures were analyzed. The classification of structural aberrations was carried out following the guidance of the International System for Cytogenetic Nomenclature (ISCN) ■ An International System for Human Cytogenetic Nomenclature (1985) ISCN 1985. Report of the Standing Committee on Human Cytogenetic Nomenclature, Birth Defects Orig. Artic. Ser., 21, (1985) 1–117 ■. 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 [21]. The assay was considered positive (compound shown genotoxic activity) when there was a statistically significant and reproducible increase in the proportion of cells with structural aberrations (excluding gaps) at any concentration compared to the negative control and it exceeded the normal range (based on internal background historic data).

Mouse lymphoma thymidine kinase assay

The method used corresponded to the fluctuation protocol [22] following the recommendations of OECD guideline 476 [23]. The cell line employed in the experiments was the L5178Y TK^{+/−} clone-3.7.2.C. Two independent experiments were performed, each conducted in the absence and presence of metabolic activation. Test concentrations for Experiment 1 were selected based on the results of a preliminary assay. Test concentrations and conditions are detailed in ► **Table 3**. Control cultures were treated with water (vehicle control), EMS or MMS (positive controls in the absence of S9, to generate large and small colonies, respectively) and 3-MC (positive control in the presence of S9 as inducer of small colonies).

Approximately 1 × 10⁷ cells were exposed in Fischer's medium supplemented with 5 % horse serum, penicillin (100 units/ml), streptomycin (100 µg/ml), sodium bicarbonate (1.125 g/ml), sodium pyruvate (0.9 mM) and pluronic acid (0.05 % w/v), to several concentrations of cizolirtine for 4 h at 37 °C in a 5 % CO₂ atmos-

► Table 1 Ames Test.

Experiment 1	Mean number revertant colonies per plate ± SD ^(a)																					
	TA98			TA100			TA1535			TA1537												
	-S9	+S9	-S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9											
Treatment (µg/plate)																						
Vehicle control	23.3 ± 1.53	34.7 ± 2.08	115.3 ± 3.51	113.7 ± 1.53	20.3 ± 1.53	15.7 ± 2.08	15.0 ± 2.00	16.3 ± 1.53	141.0 ± 1.00	143.3 ± 1.53	141.0 ± 1.00	143.3 ± 1.53	141.0 ± 1.00	143.3 ± 1.53	141.0 ± 1.00	143.3 ± 1.53	141.0 ± 1.00	143.3 ± 1.53	141.0 ± 1.00	143.3 ± 1.53	141.0 ± 1.00	143.3 ± 1.53
20.6	23.7 ± 1.53	34.0 ± 1.73	118.0 ± 2.00	113.3 ± 1.53	21.3 ± 1.53	16.0 ± 1.00	15.0 ± 1.00	16.7 ± 1.53	143.0 ± 1.00	142.3 ± 1.53	143.0 ± 1.00	142.3 ± 1.53	143.0 ± 1.00	142.3 ± 1.53	143.0 ± 1.00	142.3 ± 1.53	143.0 ± 1.00	142.3 ± 1.53	143.0 ± 1.00	142.3 ± 1.53	143.0 ± 1.00	142.3 ± 1.53
61.7	23.7 ± 1.15	35.0 ± 1.00	117.7 ± 2.52	114.3 ± 1.53	20.3 ± 1.53	16.0 ± 1.00	15.0 ± 1.01	16.3 ± 0.58	141.3 ± 1.53	140.7 ± 1.15	141.3 ± 1.53	140.7 ± 1.15	141.3 ± 1.53	140.7 ± 1.15	141.3 ± 1.53	140.7 ± 1.15	141.3 ± 1.53	140.7 ± 1.15	141.3 ± 1.53	140.7 ± 1.15	141.3 ± 1.53	140.7 ± 1.15
185.2	22.7 ± 1.53	35.0 ± 1.73	118.3 ± 2.08	113.3 ± 1.53	20.0 ± 1.00	16.7 ± 1.15	15.7 ± 1.53	16.3 ± 0.58	142.0 ± 1.00	142.7 ± 1.15	142.0 ± 1.00	142.7 ± 1.15	142.0 ± 1.00	142.7 ± 1.15	142.0 ± 1.00	142.7 ± 1.15	142.0 ± 1.00	142.7 ± 1.15	142.0 ± 1.00	142.7 ± 1.15	142.0 ± 1.00	142.7 ± 1.15
555.6	23.0 ± 1.73	35.3 ± 1.15	115.3 ± 2.52	114.3 ± 1.15	21.7 ± 1.53	16.3 ± 0.58	14.7 ± 1.53	16.0 ± 1.00	141.3 ± 0.58	141.3 ± 0.58	141.3 ± 0.58	141.3 ± 0.58	141.3 ± 0.58	141.3 ± 0.58	141.3 ± 0.58	141.3 ± 0.58	141.3 ± 0.58	141.3 ± 0.58	141.3 ± 0.58	141.3 ± 0.58	141.3 ± 0.58	141.3 ± 0.58
1666.7	23.3 ± 1.53	35.7 ± 0.58	116.3 ± 2.52	113.0 ± 1.00	21.7 ± 0.58	16.3 ± 1.53	15.7 ± 1.15	16.0 ± 1.00	142.0 ± 1.00	143.0 ± 2.00	142.0 ± 1.00	143.0 ± 2.00	142.0 ± 1.00	143.0 ± 2.00	142.0 ± 1.00	143.0 ± 2.00	142.0 ± 1.00	143.0 ± 2.00	142.0 ± 1.00	143.0 ± 2.00	142.0 ± 1.00	143.0 ± 2.00
5000	23.7 ± 1.15	35.0 ± 2.00	117.7 ± 2.08	113.3 ± 0.58	20.7 ± 1.53	15.7 ± 1.53	15.0 ± 1.00	16.0 ± 1.00	142.0 ± 1.00	143.0 ± 1.00	142.0 ± 1.00	143.0 ± 1.00	142.0 ± 1.00	143.0 ± 1.00	142.0 ± 1.00	143.0 ± 1.00	142.0 ± 1.00	143.0 ± 1.00	142.0 ± 1.00	143.0 ± 1.00	142.0 ± 1.00	143.0 ± 1.00
Positive Control ^(b)	125.0 ± 4.36	257.0 ± 10.8	300.0 ± 20.0	284.7 ± 6.03	303.3 ± 8.08	84.7 ± 8.74	54.7 ± 6.03	54.0 ± 5.00	735.0 ± 26.0	347.3 ± 17.0	735.0 ± 26.0	347.3 ± 17.0	735.0 ± 26.0	347.3 ± 17.0	735.0 ± 26.0	347.3 ± 17.0	735.0 ± 26.0	347.3 ± 17.0	735.0 ± 26.0	347.3 ± 17.0	735.0 ± 26.0	347.3 ± 17.0
Experiment 2	Mean number revertant colonies per plate ± SD ^(a)																					
Experiment 2	TA98			TA100			TA1535			TA1537												
	-S9	+S9	-S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9											
	-S9	+S9	-S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9											
Treatment (µg/plate)																						
Vehicle control	30.7 ± 2.52	33.3 ± 1.53	116.7 ± 1.53	119.7 ± 3.06	20.3 ± 1.53	16.7 ± 1.53	14.7 ± 0.58	22.0 ± 1.00	143.0 ± 1.00	141.0 ± 1.00	143.0 ± 1.00	141.0 ± 1.00	143.0 ± 1.00	141.0 ± 1.00	143.0 ± 1.00	141.0 ± 1.00	143.0 ± 1.00	141.0 ± 1.00	143.0 ± 1.00	141.0 ± 1.00	143.0 ± 1.00	
20.6	29.3 ± 0.58	33.7 ± 2.08	117.7 ± 1.53	120.0 ± 2.00	20.0 ± 1.73	16.0 ± 1.00	14.7 ± 0.58	21.7 ± 1.53	141.7 ± 2.08	141.3 ± 1.53	141.7 ± 2.08	141.3 ± 1.53	141.7 ± 2.08	141.3 ± 1.53	141.7 ± 2.08	141.3 ± 1.53	141.7 ± 2.08	141.3 ± 1.53	141.7 ± 2.08	141.3 ± 1.53	141.7 ± 2.08	
61.7	29.0 ± 1.73	34.0 ± 2.00	116.3 ± 1.53	119.0 ± 1.00	20.0 ± 1.00	16.3 ± 1.53	15.3 ± 0.58	22.3 ± 1.15	141.7 ± 1.53	140.7 ± 1.15	141.7 ± 1.53	140.7 ± 1.15	141.7 ± 1.53	140.7 ± 1.15	141.7 ± 1.53	140.7 ± 1.15	141.7 ± 1.53	140.7 ± 1.15	141.7 ± 1.53	140.7 ± 1.15	141.7 ± 1.53	
185.2	30.3 ± 0.58	34.3 ± 2.08	117.7 ± 1.53	120.0 ± 2.65	19.7 ± 2.08	16.7 ± 1.15	15.7 ± 0.58	21.3 ± 1.53	141.3 ± 1.53	140.0 ± 1.73	141.3 ± 1.53	140.0 ± 1.73	141.3 ± 1.53	140.0 ± 1.73	141.3 ± 1.53	140.0 ± 1.73	141.3 ± 1.53	140.0 ± 1.73	141.3 ± 1.53	140.0 ± 1.73	141.3 ± 1.53	140.0 ± 1.73
555.6	30.3 ± 1.00	33.7 ± 2.08	116.7 ± 0.58	119.0 ± 1.00	20.0 ± 1.00	16.7 ± 0.58	15.0 ± 0.00	21.7 ± 1.15	142.0 ± 1.00	141.0 ± 1.00	142.0 ± 1.00	141.0 ± 1.00	142.0 ± 1.00	141.0 ± 1.00	142.0 ± 1.00	141.0 ± 1.00	142.0 ± 1.00	141.0 ± 1.00	142.0 ± 1.00	141.0 ± 1.00	142.0 ± 1.00	
1666.7	29.0 ± 1.73	34.0 ± 1.00	116.3 ± 1.53	118.7 ± 0.58	20.3 ± 1.53	16.3 ± 0.58	15.7 ± 0.58	22.3 ± 0.58	142.3 ± 1.53	140.3 ± 1.15	142.3 ± 1.53	140.3 ± 1.15	142.3 ± 1.53	140.3 ± 1.15	142.3 ± 1.53	140.3 ± 1.15	142.3 ± 1.53	140.3 ± 1.15	142.3 ± 1.53	140.3 ± 1.15	142.3 ± 1.53	
5000	30.7 ± 0.58	32.7 ± 2.08	117.3 ± 1.53	119.0 ± 1.00	20.3 ± 0.58	16.0 ± 1.00	15.0 ± 0.00	20.7 ± 0.58	141.0 ± 1.00	140.3 ± 1.15	141.0 ± 1.00	140.3 ± 1.15	141.0 ± 1.00	140.3 ± 1.15	141.0 ± 1.00	140.3 ± 1.15	141.0 ± 1.00	140.3 ± 1.15	141.0 ± 1.00	140.3 ± 1.15	141.0 ± 1.00	
Positive Control ^(b)	95.7 ± 7.57	212.0 ± 17.6	324.0 ± 14.4	276.0 ± 7.00	393.0 ± 7.55	64.3 ± 6.11	73.7 ± 8.74	50.3 ± 3.06	756.0 ± 51.1	363.0 ± 4.58	756.0 ± 51.1	363.0 ± 4.58	756.0 ± 51.1	363.0 ± 4.58	756.0 ± 51.1	363.0 ± 4.58	756.0 ± 51.1	363.0 ± 4.58	756.0 ± 51.1	363.0 ± 4.58	756.0 ± 51.1	363.0 ± 4.58

^(a)Mean of 3 replicas for all tested conditions. ^(b)In absence of S9, TA98: 2NF (1 µg/plate); TA100 and TA1535: NaN3' (1 µg/plate); TA1537: AAC (50 µg/plate); *E. coli* WP2 *uvrA* (pKM101): 4NQ (1 µg/plate). In presence of S9, AAN. All strains (1 mg/plate).

► **Table 2** Chromosome aberration assay.

Structural aberrations														
Treatment	Concentration (µg/ml)	S9	Exposure -recovery (h)	Cells scored	Mit. Index	Gaps	Chromosome			Chromatid			Cells with aberrations	
							Deletion	Exchange	Deletion	Exchange	Deletion	Exchange	Incl. Gaps	Excl. Gaps
Vehicle	-	-	24+0	400	100	9	1	0	10	0	19	11		
Cizolirtine	115	-	24+0	200	78	5	0	0	0	0	5	0		
	720	-	24+0	200	51	1	1	2	0	0	4	3		
	1800	-	24+0	200	35	3	0	3	0	0	6	3		
EMS	250	-	24+0	200	76	13	6	52	9	68***	57***			
Vehicle	-	+	3-21	400	100	4	1	0	6	0	10	6		
Cizolirtine	288	+	3-21	200	82	5	0	0	0	0	5	0		
	1800	+	3-21	200	81	3	1	3	0	0	7	4		
	4500	+	3-21	200	52	2	5	3	0	0	9	7		
CP	4	+	3-21	200	66	8	4	60	13	63***	58***			
Structural aberrations														
Treatment	Concentration (µg/ml)	S9	Exposure -recovery (h)	Cells scored	Mit. Index	Gaps	Chromosome			Chromatid			Cells with aberrations	
							Deletion	Exchange	Deletion	Exchange	Deletion	Exchange	Incl. Gaps	Excl. Gaps
Vehicle	-	-	24+0	400	100	1	0	0	2	1	4	3		
Cizolirtine	200	-	24+0	200	97	3	1	0	3	0	7*	4		
	400	-	24+0	200	76	1	0	1	0	0	2	1		
	1600	-	24+0	200	34	4	1	4	0	0	8*	5		
EMS	250	-	24+0	200	72	14	2	48	7	56***	49***			
Vehicle	-	+	3-21	400	100	2	4	3	0	0	7	5		
Cizolirtine	1125	+	3-21	200	102	0	1	3	1	5	5			
	2250	+	3-21	200	80	2	1	1	0	4	2			
	4500	+	3-21	200	97	2	0	4	0	6	4			
CP	4	+	3-21	200	75	13	4	61	5	62***	54***			
Vehicle	-	-	48+0	400	100	2	2	0	0	4	2			
Cizolirtine	1600.0	-	48+0	200	38	2	1	3	0	6	4			
Vehicle	-	+	3+45	400	100	2	0	1	0	3	1			
Cizolirtine	4500.0	+	3+45	200	95	2	0	0	0	2	0			

* p<0.05; *** p<0.001; Fisher's exact test. CP: Cyclophosphamide; EMS: Ethyl methanesulfonate.

► **Table 3** Mouse Lymphoma assay.

Experiment 1		-S9				+S9			
Treatment	Concentration (µg/ml)	% RTG	Mutant frequency ^(a)	Treatment	Concentration (µg/ml)	% RTG	Mutant frequency ^(a)		
Vehicle	-	100	34.3±1.70	Vehicle	-	100	33.5±5.45		
Cizolirtine	1250	88.5±4.95	39.0±2.83	Cizolirtine	1250.0	93.5±27.58	33.0±4.24		
	1500	73.5±6.36	32.0±2.83		1500.0	78.0±14.14	32.0±1.41		
	1750	73.5±0.71	34.5±0.71		1750.0	94.0±15.56	35.5±0.71		
	2000	65.5±2.12	37.0±4.24		2000.0	95.5±2.12	42.0±14.1		
EMS	250	48.5±3.53	420.5±75.7	3-MC	2.5	28.0±4.24	363.0±5.66		
MMS	15	8.0±0.00	344.5±78.5						
Experiment 2		-S9				+S9			
Treatment	Concentration (µg/ml)	% RTG	Mutant frequency ^(a)	Treatment	Concentration (µg/ml)	% RTG	Mutant frequency ^(a)		
Vehicle	-	100	35.5±4.20	Vehicle	-	100	36.3±2.99		
Cizolirtine	356	48.5±2.12	35.5±4.95	Cizolirtine	633	76.0±4.24	35.5±3.53		
	475	34.5±0.71	37.5±2.12		844	56.5±4.95	34.5±4.95		
	633	22.0±1.41	47.5±10.6		1125	27.5±2.12	41.5±6.36		
	844	12.0±1.41	50.0±9.90		1500	13.0±1.41	44.0±2.83		
	1125 ^(b)			2000 ^(b)					
EMS	250	65.0±0.00	335.5±60.1	3-MC	2.5	31.5±6.36	460.0±65.0		
MMS	10	33.5±3.53	263.0±11.3						

^(a) Per 10⁶ viable cells. ^(b) Excluded due to excessive toxicity. EMS: Ethyl methanesulfonate. MMS: Methyl methanesulfonate. 3-MC: 3-methylcholanthrene.

► **Table 4** Mouse bone marrow 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	1.38 ± 0.46	1.9 ± 1.4	1.8 ± 1.5
	Cizolirtine	100	10	1.12 ± 0.24	2.9 ± 2.1	1.6 ± 1.0
		175	10	1.40 ± 0.26	1.8 ± 1.9	2.0 ± 1.9
		300	10	1.19 ± 0.36	2.2 ± 2.3	0.8 ± 0.8
	CP	50	10	0.65 ± 0.11 ***	28.3 ± 10.0 ***	2.1 ± 1.5
48 h.	Vehicle	–	10	1.44 ± 0.82	1.7 ± 1.3	1.0 ± 0.7
	Cizolirtine	100	10	1.56 ± 0.66	1.1 ± 1.1	1.3 ± 1.6
		175	10	1.12 ± 0.25	1.1 ± 1.5	1.1 ± 1.2
		300	10	1.39 ± 0.47	1.8 ± 1.6	1.5 ± 1.4
72 h.	Vehicle	–	10	1.13 ± 0.41	1.9 ± 1.3	1.6 ± 1.6
	Cizolirtine	100	10	1.26 ± 0.54	1.8 ± 1.2	1.4 ± 1.3
		175	10	0.92 ± 0.30	2.2 ± 1.5	2.0 ± 1.7
		300	10	1.17 ± 0.35	1.5 ± 1.8	1.3 ± 1.1

*** p < 0.001 Dunnett's test.

phere. Duplicate cultures were set up for the vehicle control and each test substance concentration, and single cultures for the positive control. After the 4 h treatment cells were washed with tissue culture medium, resuspended in 10 % serum-supplemented Fischer's medium, and, when appropriate, the cell concentration was adjusted to 3×10^5 cells/ml. For the viability assay, each culture was diluted to 2×10^3 cells/ml. From each culture, 3 samples of 0.1 ml of this dilution were each added to 25 ml of cloning medium and poured into a 90 mm Petri dish, so giving 200 cells per plate. For the mutant selection assay, trifluorothymidine (TFT) stock solution was added to cloning medium, to a final concentration of 3 µg/ml. Triplicate samples of 1×10^6 cells each were suspended in 25 ml cloning medium containing TFT and poured into 90 mm Petri dishes. Plates were incubated for 14 days at 37 °C in a 5 % CO₂ atmosphere. The colonies were then counted usually using an Artek 880 automated colony counter. If there were small colony type mutants present that were below 0.1 mm (size corresponding to the resolution limit of the equipment), all mutant selection plates were counted by eye. Large and small colonies were scored for vehicle, positive controls and for test substance concentrations showing a significant increase in mutant frequency. The percentage relative survival (RTG) for each treatment condition compared to the vehicle-control culture and the mutant frequency per 10⁶ viable cells (MF) were determined. The vehicle-control log mutant frequency was compared with that from each treatment concentration by means of the Dunnett's test [18] and checked for a linear trend by chi-square analysis, according to UKEMS guidelines [24]. The result was considered positive when the mutation frequency of any test concentration exceeded the sum of the mean control mutant frequency plus the global evaluation factor (90 mutants per 10⁶ viable cells for the agar version of the test [25]) and there was a statistically significant dose-response.

Mouse bone marrow micronucleus test

The micronucleus test was conducted using the method previously described by Schmid [26] following the recommendations of the

OECD 474 guideline [27]. Albino Swiss CFLP mice were supplied by Interfauna Ibérica (Sant Feliu de Codines, Spain). The animals were approximately 9–10 weeks old at the moment of administration showing a weight range of 26.7–38.9 g for males and 22.0–32.0 g for females. Animals were allocated in cages in groups of maximum 5, separated by sex. Animals had *ad libitum* access to water and commercial rodent diet (Panlab S.L.). The night prior to administration of the test substance mice were kept under fasting conditions. Based on a preliminary study, dose levels of 100, 175 and 300 mg/kg of cizolirtine were selected for treatment. Groups of 15 male and 15 female animals each received either saline (vehicle) or cizolirtine at the previously mentioned doses. A positive control group of 5 males and 5 females received CP at a dose of 50 mg/kg. All animals were dosed by intraperitoneal route of administration (dose volume of 10 ml/kg). Groups of 5 male and 5 female mice were sacrificed by cervical dislocation 24, 48 or 72 h after vehicle or cizolirtine administration. Mice treated with positive control were sacrificed 24 h post-administration (► **Table 4**). For each animal, bone marrow was extracted from dissected femurs and smears were prepared on slides containing a drop of foetal calf serum (two slides per animal). The smears were fixed with methanol before being stained with a 5 % Giemsa solution. Prior examinations under light microscopy, the slides were coded to avoid any bias during the analysis. For each individual, the relative proportion of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) was noted by counting a total sample of 1000 erythrocytes. On the other hand, the number of micronucleated polychromatic erythrocytes (MNPCE) per 2000 PCE was also calculated. The number of MNPCE in belonging to each treated group (either, combined or males and females separately) were compared versus the number obtained in the vehicle group using the Dunnett's test [18] 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.

Results

Ames Test

In the two independent conducted assays, no increases in the number of revertants compared to the vehicle control were observed when cizolirtine was tested in any of the five strains composing the bacterial battery. The same result was observed in presence and absence of a rat liver metabolic activation system (S9) up to concentrations of 5000 µg/plate. Results are shown in ► **Table 1**. There was neither, evidence of precipitation of cizolirtine nor signs of cytotoxicity at any of the six concentrations assayed and in any of the different assayed conditions. Positive controls employed in the assay clearly increased the number of revertants in comparison to the vehicle control. As a consequence, it was concluded that cizolirtine was not showing any genotoxic effect in this bacterial assay.

Human lymphocyte chromosome aberration assay

Human peripheral blood lymphocytes from two different donors were exposed *in vitro* to cizolirtine at concentrations of up to 1800 and 4500 µg/ml in the absence and in the presence of metabolic activation, respectively. The concentration of 4500 µg/ml equals to 10 mM. Results of mitotic index evaluation are shown in ► **Table 2**. In both experiments, it was observed a decrease in mitotic index with increasing concentrations of the test substance. Concentrations for metaphase analysis were selected trying to include concentrations showing an overall reduction in mitotic index.

Likewise, in both experiments, all cizolirtine-treated cultures showed a frequency of cells with chromosome aberrations excluding gaps similar to that of the vehicle control. In the second experiment, in the 24-h sampling but including gaps, the 200 and 1600 µg/ml concentrations in the absence of S9 showed a slight statistical increase in the frequency of cells with chromosome aberrations ($p < 0.05$), as compared to the vehicle control value. Nevertheless such increases were not considered biologically significant since they were moderate, were within the laboratory historical control values, were not observed at similar concentrations in the first assay, were not reproduced at a latter sampling time (48 h) in the second assay, and did not translate into increased frequency of cells with chromosome aberrations when excluding gaps. On the other hand, ethylmethanesulphanate and cyclophosphamide caused in absence and presence of S-9 mix, respectively, statistically significant increases ($p < 0.001$) in the frequency of cells with chromosome aberrations, as compared to the vehicle control value. Overall, the results obtained allow concluding that cizolirtine shows no signs of clastogenic activity in this *in vitro* experimental system.

Mouse Lymphoma assay

The preliminary cytotoxicity test conducted with cizolirtine showed that this compound caused complete toxicity at a concentration of 2500 µg/ml when dissolved in water. Moreover, it was observed that the test compound was inducing a reduction in pH (noted on dosing by colour change in the phenol red indicator of the culture media), and corroborated after measuring the value at the end of the exposure period. The degree of the pH decrease (estimated by colour change in the phenol red indicator) indicated that the results could have been obtained at concentrations producing a pH shift greater than 1 unit. It has been published that pH shifts great-

er than 1 unit can rise false positive effects [28]. To avoid this pH limiting factor, a second preliminary cytotoxicity test was carried out. In this occasion, cizolirtine formulation was neutralised with sodium hydroxide, showing that the compound was even more cytotoxic when neutralised. In these later tests, cizolirtine was dissolved and diluted in tissue culture medium.

Based on the preliminary tests, 4 independent mutation assays were conducted (2 in the absence and 2 in the presence of metabolic activation) (► **Table 3**). First and second mutation assays (Experiment 1) used concentrations of cizolirtine ranging from 1250 to 2000 µg/ml without pH neutralisation in the absence and presence of S9 mix (these concentration levels causing not significantly more than 1 unit of shift in pH). Third and fourth assays (Experiment 2) were performed in neutralising conditions of pH. More precisely, third assay used a compound range of concentrations between 356 and 844 µg/ml (in the absence of S9 mix) while in the presence of metabolic activation the range used was 633–1500 µg/ml.

In the first and second mutation assays, the maximum dose levels were limited by the pH decrease caused by the test material (large shifts in pH should be avoided due to potential false positive effects). The highest concentrations in the third and fourth assays induced a reduction in the mean relative total growth (parameter equivalent to survival) to 12% and 13% in the absence and presence of S9 mix, respectively. This represents a definitive level of toxicity. Sensitivity of the experiments was proved by the well performance of both positive controls and the effectiveness of the S9 mix. No evidence of mutagenic activity was observed in cizolirtine-treated cultures in any experimental condition tested in mouse lymphoma L5178Y cells.

Bone marrow mouse micronucleus test

Clinical signs and mortality were observed among animals treated with cizolirtine. More precisely, two male and two female animals treated with 300 mg/kg died after cizolirtine administration. These animals were replaced by animals from a supplementary group treated with the same dose. This way, data was obtained from 10 animals per group of treatment (5 males and 5 females). Diminished spontaneous activity was observed in all animals treated with cizolirtine, as well as tremor in intermediate- and high-dose treated animals. High-dose animals treated at 300 mg/kg also showed excitation, clonic convulsions, vocalization and slight ataxia. The first signs were detected 5 min after administration. All animals had a normal outward appearance 3 h after administration. Cizolirtine treated groups exhibited at all sampling time points, a MNPCE frequency similar to that of the vehicle control group (► **Table 4**). On the other hand, the administration of 50 mg/kg of cyclophosphamide caused a statistically significant ($P < 0.001$) increase in the MNPCE frequency with respect to the vehicle control group at 24 h post-administration. Likewise, samples extracted from treated groups exhibited a PCE/NCE ratio similar to that of control group. This ratio showed a statistically significant decrease ($p < 0.001$) in animals treated with cyclophosphamide. The results obtained in this *in vivo* test allow concluding that cizolirtine shows no evidence of genotoxic potential or cytotoxic effects on the bone-marrow of mice when administered intraperitoneally as a single dose.

Discussion

Cizolirtine is a modulator of the release of substance P and calcitonin gene-related peptide (CGRP) showing activity in the treatment of pain and urinary incontinence. As part of the preclinical safety-assessment program required by the regulatory authorities, the genotoxic potential of cizolirtine was evaluated employing a battery of *in vitro* and *in vivo* genetic toxicity assays according to the recommendations of ICH and OECD guidelines [17, 20, 23, 27, 29]. This package consisted in an Ames test, a chromosome aberration assay in Human Lymphocytes, a Mouse Lymphoma gene mutation assay and a single dose mouse micronucleus assay. Cizolirtine demonstrated to be devoid of genotoxic potential in the Ames test, Mouse Lymphoma assay and the mouse bone marrow micronucleus test. In the Human Lymphocyte chromosome aberration, cizolirtine-treated cultures showed no increases in the number of cells with chromosome aberrations excluding gaps in any of the assays and any of the treatment conditions. When including gaps, a slight but statistically significant ($p < 0.05$) increase in the frequency of chromosome aberrations was observed in experiment 2 at 24 h in the absence of metabolic activation in the high and in the low concentrations analysed in the assay, but not in the intermediate concentration. In other words, there was not a dose related effect associated with this response. Despite this increase was statistically significant, it was within the laboratory historical control value range and more importantly, the same effect was neither observed in the analysis made at a later time point (48 h) in the same experiment nor under similar conditions in the experiment 1. An important point to take in consideration is that gaps are not equivalent to chromosome breaks as they are considered non-staining regions of a single chromatid in which there is minimal misalignment of the chromatid [20], and are considered to represent spiral unravellings but not chromosome aberrations [30]. As such, and according to the current regulatory guidance, in the interpretation of chromosomal aberration study outcomes, gaps have to be recorded and reported separately but not included in the total aberration frequency [20]. Chromosomal study results are therefore *a priori* judged based on total chromosomal aberration frequencies excluding gaps. In this respect, cizolirtine did not show any increase in total chromosomal aberration frequencies excluding gaps in any of the conducted experiments and under any of the tested conditions, and was therefore considered as non-genotoxic in this experimental system. But additionally, the lack of genotoxic effects of cizolirtine in the Mouse Lymphoma assay (that allows detecting the effect of chromosome aberrations in mammalian cells *in vitro*) and in the bone marrow mouse micronucleus assay (that detects chromosome aberrations *in vivo*) further supports that the slight increase in the frequency of chromosome aberrations including gaps observed in the Human Lymphocyte chromosome aberration assay would be lacking of biological significance.

Cizolirtine contains a diarylalkylamine chemical substructure that for a number of compounds has been linked to the potential for inducing chromosome damage [31, 32]. One of these compounds is the antihistaminic drug diphenhydramine, which is a close structural analogue. Positive results have been reported for diphenhydramine in the chromosomal aberrations test in CHO cells in the absence of metabolic activation but not in the presence of S9 [31, 32]. However, cizolirtine show neither clastogenic nor an-

eugenic activities in the chromosome aberrations test using human lymphocytes, in the mouse lymphoma assay or in the *in vivo* micronucleus test depicted in this genotoxicity assessment.

Taking in mind the results obtained with the whole battery of studies conducted, the conclusion reached is that cizolirtine would not pose a genotoxic risk when administered in humans.

Conflict of Interest

The authors were employees of Esteve Pharmaceuticals.

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