Genotoxic and aneugenic properties of an imidazole derivative

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ABSTRACT: To contribute to a more accurate characterization of the mutagenic and aneugenic effects of thiabendazole (TBZ), a widely used antiparasitic and food preservative drug, the induction of sister chromatid exchanges (SCEs) and mitotic spindle anomalies as cytogenetic end-points were investigated. Studies were carried out in Chinese hamster ovary (CHO) cells and human peripheral blood lymphocytes. A significant dose-dependent increase in SCE frequency was observed in CHO cells with S9-Mix ($P < 0.01$) in the 50–100 µg ml^{-1} dose-range, while in the absence of S9-Mix, an enhancement of the SCE frequency was exhibited at the highest dose ($P < 0.01$). In CHO-K1 cells a significant increase in mitotic spindle anomalies ($P < 0.01$) was observed with the highest concentration assayed reflecting the specific effect of TBZ formulation at the microtubule level. Cell proliferation kinetics (CPK) were not modified by the addition of this pharmaceutical product. In human lymphocyte cultures, exposure to 100 µg ml^{-1} TBZ formulation resulted in a significant decrease of the mitotic index (MI) ($P < 0.003$) and changes in the replication index (RI) ($P < 0.05$).

KEY WORDS: thiabendazole; human lymphocytes; cytogenetic endpoints; CHO cells; mitotic index; sister chromatid exchanges; cell proliferation kinetics; mitotic spindle disturbances; aneugen

Introduction

The 2-(4′-thiazolyl) benzimidazole, known as thiabendazole (TBZ), is a broad spectrum antihelminthic chemical used worldwide in the treatment of human and animal parasitism. It is also used in the preservation of food for breeding animal species, as well as in postharvest treatment to preserve citrus fruits during transport and storage (Reygrobellet *et al*., 1996; Sasaki *et al*., 1997). The introduction of TBZ represented a breakthrough in the therapy of cutaneous *Larva migrans* and *Strongyloide stercoralis* infection (Davies *et al*., 1993). Furthermore, it is also effective in gastrointestinal nematode infections as well as in early trichinosis (Goodman and Gilman, 2001).

Several studies have analysed the genotoxic effect of TBZ on different prokaryotic and eukaryotic systems (Table 1). Nakagawa and Moore (1995) described the cytotoxic effect of TBZ on isolated rat hepatocytes. However, contradictory results were reported when micronuclei induction was used as a cytogenetic endpoint. Natarajan (1993) observed that TBZ induced kinetocore-negative micronuclei in human fibroblasts, whereas Van Hummelen *et al*. (1995) reported that human lymphocytes were negative for the induction of micronuclei with and without the presence of S9-Mix. No enhancement in micronuclei frequency was observed in human lymphocyte cultures after *in vitro* TBZ treatment (Holden *et al*., 1980; Adler *et al*., 1991; Migliore and Neri, 1991; Watanabe-Akanuma *et al*., 2005). Negative results were also reported by Adler (1993) for bone marrow cells from different mice strains and mice spermatocytes (Miller and Adler, 1992). Moreover, previous observations suggested a clastogenic behavior of TBZ evidenced by an increased frequency of bridges, lagged chromosomes and SCEs in Chinese hamster ovary (CHO) treated-cells (Mudry *et al*., 1986; 1987; 1995).

It is well known that the frequency of cells carrying chromosomal alterations does not always agree with the real damage inflicted on the cellular population. In most cases the analyses are performed only in mitotic cells without taking into account that damaged cells can die

Table 1. Principal positive results of TBZ genotoxic studies

Test system	Genetic effect	References
Fungi and yeast	Aneuploidy	Parry and Sors, 1993
Aspergillus nidulans	Aneuploidy	Kappas *et al.*, 1974
Rat cells	C-mitotic effects and aberrant mitosis	Styles *et al.*, 1973
Chinese hamster ovary cell line (CHO)	Chromosome aberrations	Mudry *et al.*, 1986
	SCE	Mudry *et al.*, 1987
Chinese hamster ovary cell line (CHO)	Anaphase bridges and multipolar spindles	Natarajan *et al.*, 1993
Chinese hamster primary embryonic cells	Spindle disturbances	Warr *et al.*, 1993
CHO embryonic cells	Micronuclei	Mailhes *et al.*, 1997
Mouse oocytes	Aneuploidy	Pacchierotti *et al.*, 1982
Mouse spermatocytes	Aneuploidy	Leopardi *et al.*, 1993
Rat hepatocytes	Cytotoxicity	Nakagawa and Moore, 1995
Mouse organs	Comet assay	Sasaki *et al.*, 1997
Human lymphocytes	Micronuclei Cyt-B	Van Hummelen *et al.*, 1995
Human HepG$_2$ and lymphoblastoid cell line	Induction of Cyt P450 1A1	Delescluse *et al.*, 2001
Human lymphocytes	Micronuclei Cyt-B	Ramirez *et al.*, 2000
	Aneuploidy	
Saccharomyces pombe	Defective chromosome segregation	Silverstein *et al.*, 2003

before entering mitosis or are unable to progress through the cell-cycle thus failing to enter the M phase.

In order to obtain a more accurate characterization of the deleterious effect of an imidazole derivative a new method was developed using *in vitro* analysis of spindle apparatus modifications in relation to cell proliferation kinetics, mitotic index and SCE induction.

Materials and Methods

Chemicals

Thiabendazole was commercially obtained as Foldan®, Andrómaco (thiobendazole 500 mg; Excipients c.s.). The assayed TBZ was prepared using NaCl solution (9%) as the vehicle, in a final concentration of 10 mg ml^{-1}. TBZ solution was added to cultures so that the addition of 100 µl allowed them to reach concentrations of 50 and 100 µg ml^{-1} of TBZ.

Lymphocyte Cultures from Human Peripheral Blood

Heparinized blood samples were obtained from ten healthy adult donors of both sexes with no recent history of exposure to mutagens. Duplicate peripheral blood cultures were prepared as follows: 1 ml of whole blood was placed in sterile tubes containing 7 ml of RPMI 1640 medium supplemented with 15% of fetal bovine serum (Gibco), 0.1 ml of PHA (Gibco) and 0.1 ml of 5-bromo-2-deoxyuridine (BrdU, 10 µg ml^{-1}) (Sigma) and the cells were harvested after a 72 h incubation at 37 °C.

Negative controls (untreated cells and solvent-vehicle-treated cells) were performed and run simultaneously with TBZ formulation-treated cultures. None of the treatments produced significant pH changes in the culture medium. One hour before harvesting, 0.2 ml of Colcemid (10 µg ml^{-1}, Sigma) was added to each culture flask. Afterwards, the cells were harvested and fixed by conventional methods. Microscopic preparations were stained following a modified fluorescence plus Giemsa (FPG) technique (Perry and Wolff, 1974).

Chinese Hamster Ovary (CHO) Cell Cultures

CHO cells (1×10^{-6}) were cultured and treated continuously with TBZ formulation through a concentration range of 50–100 µg ml^{-1}. The cells were then incubated with Ham F12 medium (Gibco) plus 5 µg ml^{-1} BrdU (Sigma), and the drug was added 6 h after seeding with a total culture time of 36 h. To study chromosomal aberrations, colcemid (2×10^{-7} M final concentration) was added for the last 2 h of culture. The cells were treated for 30 min with 0.075 M KCl to spread the chromosomes and then were fixed in methanol–acetic acid (3:1, v/v). Drops of fixed cells were placed in slides and allowed to air dry. Slides were then stained for 10–15 min, as required in 3% Giemsa (Sigma).

A metabolic activation system (S$_9$-Mix) was obtained from adult male Wistar rats treated with phenobarbital and naphthoflavone for 3 days by intraperitoneal route (Matsushima *et al.*, 1976). Preparation of the S9 fraction was based on the procedure of Garner *et al.* (1972). The freshly excised livers were placed in pre-weighed beakers containing approximately 1 ml of chilled 0.15 M KCl g^{-1} of wet liver. One rat liver weighs approximately 10–15 g. After being weighed, the livers were washed several times in fresh chilled KCl. Successive washes in KCl are essential to ensure a sterile preparation and to remove hemoglobin which can inhibit the activity of cytochrome P450 enzymes. The washed livers were transferred to a beaker containing 3 volumes of 0.15 M KCl (3 mg g^{-1} wet liver), minced with sterile

scissors, and homogenized in a Potter–Elvehjem apparatus with a teflon pestle or with a Polystrom homogenizer. The homogenate was centrifuged for 10 min at 9000 g (8700 rpm) and the supernatant (the S9 fraction) was decanted and saved. The sterility of the preparation was determined by plating 0.1 ml on minimal agar containing histidine and biotin. The freshly prepared S9 fraction was distributed in 1–2 ml portions, frozen quickly and stored at −18 °C.

Cells were exposed to the extract and to the S9 mix (10 ml of the mixture was prepared with the following composition: 20 mM Hepes buffer (pH = 7.2) 2 ml; 50 mM MgCl$_2$, 1 ml; 330 mM KCl, 1 ml; 50 mM glucose-6-phosphate, 1 ml; 40 mM NADP, 1 ml; distilled water, 1 ml; S9 fraction, 3 ml) for 2 h at 37 °C. The cells were then grown in Ham's F10 medium supplemented with 15% fetal bovine serum for 24 h (Carballo *et al*., 1992). Cultures with complete medium and S$_9$-Mix without TBZ were used as negative control and cultures were treated with 0.2 mg ml^{-1} of cyclophosphamide (CP) plus S$_9$-Mix as a positive control.

Sister Chromatid Exchanges (SCE)

The frequency of SCE was determined by analysis of 50 (46 centromeres each) and 30 second mitoses from human lymphocyte and CHO cultures (22 ± 2 centromeres each). The results were expressed as the frequency of SCE per cell in HSA and SCE per chromosome in CHO cells.

Mitotic Index (MI)

The mitotic index was estimated as the proportion of mitotic cells in 2000 cells counted for each preparation, donor and concentration (Miller and Adler, 1989).

Cell Proliferation Kinetics (CPK)

The proportion of first (M$_1$), second (M$_2$) and third (M$_3$) division cells was scored in 100 consecutive metaphases from each duplicate 72 h culture for each experimental point (Guglielmi *et al*., 1982).

Replication index (RI) was calculated as: RI = (M$_1$ × 1) + (M$_2$ × 2) + (M$_3$ × 3)/100.

Cell Cultures and Test Compound Treatment for Analysis of the Spindle Apparatus

Chinese hamster ovary cells (CHO-K1, ATCC CCL-61 from the American Type Culture Collection (Rockville, MD, USA) were grown in Ham's F10 medium (Gibco)

supplemented with 10% fetal calf serum (Gibco), 100 units ml^{-1} of penicillin (Gibco) and 10 µg ml^{-1} of streptomycin (Gibco). Experiments were set up with cultures in the log phase of growth. The cells were seeded onto sterile glass slides in 20 cm diameter culture dishes at a density of 5 × 10^4 cells ml^{-1}. Treatments with test compounds were performed 24 h after plating. TBZ formulation was used at a final concentration of 0, 50, 100 and 200 µg ml^{-1}. Negative controls (untreated cells and solvent-vehicle-treated cells) were performed and run simultaneously with TBZ formulation-treated cultures. None of the treatments produced significant pH changes in the culture medium. Cultures were incubated (37 °C, 5% CO$_2$) under a safety light for 24 h until harvesting. Cultures were trebled for each experimental point. The same batches of culture medium, sera and reagents were used throughout the study.

Fluorochrome-mediated Viability Test

At the end of the culture period, the cell viability was determined using the ethidium bromide/acridine orange assay described elsewhere (McGahon *et al*., 1995). Briefly, slides were washed twice in PBS and then 20 µl of a 1 : 1 fresh mixture of ethidium bromide (100 µg ml^{-1}, Sigma) and acridine orange (100 µg ml^{-1}, Sigma) was spread onto the cells. Then, slides were analysed using an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. At least 1000 cells were analysed at each experimental point immediately after staining in random order with regard to treatment, and the results were expressed as percentages of viable cells among all cells.

Slide Preparation and Direct Immunofluorescence Spindle Apparatus Labelling

At the end of the culture period, the culture medium was removed and the cells were fixed in methanol cooled to −20 °C. After 10 min, the methanol was discharged, the cells were rinsed twice for 10 s with cold acetone, rehydrated in PBS for at least 30 min at room temperature, and then treated as described in detail elsewhere (Soloneski *et al*., 2003). Briefly, the slides were incubated with mouse fluorescein isothiocyanate (FITC)-conjugated anti-β tubulin monoclonal antibody (Sigma) (60 min, 37 °C) in humidified atmosphere. The antibody was diluted 1 : 50 in PBS containing 1% bovine serum albumin (Sigma). Subsequently, slides were rinsed with PBS (3 times, 5 min each) and air-dried at room temperature, stained with propidium iodide (0.1 µg ml^{-1}, 10 min, Sigma) and finally mounted with an antifading medium (Vectashield mounting medium H1000; Vector Laboratories, Burlingame, CA, USA). Slides were coded

and scored blind by one researcher using an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination and CarioFISH 1.2 software (Bioanalítca Argentina, Argentina).

Analysis of Spindle Apparatus Anomalies

Criteria and analysis of the spindle anomalies was performed according to Kochendörfer *et al*. (1996) with minor modifications. Briefly, abnormal mitotic figures were classified, according to the spindle morphology and the chromatin distribution, into two major groups: multipolar and degenerated. Mitosis was defined as multipolar when it showed more than two spindle poles, independent of other aberrant structures such as disoriented spindle fibres. The multipolar spindles were divided into two subgroups: symmetrical and asymmetrical. The poles in the former subgroup were equal in size, symmetrically distributed within the cell, and the angles between poles were equal. The poles in the latter subgroup were equal in size but showed different angles between each other or a linear arrangement. Asymmetrical types were also found with poles of unequal size. A spindle apparatus was defined as degenerated when it showed an incomplete formation or disorientation of the spindle fibres by the spindle apparatus being bipolar. A spindle apparatus which seemed to have a normal spindle structure, but showed disorientation of chromatin or revealed normal chromatin distribution but disoriented fibres, was also classified as degenerated. Monopolar spindles and spindles with disorganized fibres were also classified as degenerated. A minimum of 500 mitotic spindles per sample was analysed to determine the percentage of normal and abnormal mitotic figures and the type of anomalies. The data were expressed as the frequency of normal/abnormal spindle apparatus in 100 mitotic observed spindles.

Statistical Analysis

Student's *t*-test and the Kruskall-Wallis test for ANOVA were used to test for differences among the experiments performed according to description detailed in each of the following data sets.

Student's *t*-test was used to compare the mean values of spindle anomalies between treated and control cells (Zar, 1984).

Results

In the absence of S9-Mix, a significant increase in the SCE frequency was observed in those CHO cells exposed only to the highest TBZ formulation dose ($P < 0.05$). On

Table 2. *In vitro* determination of sister chromatid exchanges (SCE) and replication index (RI), in CHO cell line exposed to TBZ with and without S_9-Mix

TBZ concentration ($\mu g\ ml^{-1}$)	SCE/chrom $X \pm SE$	CPK			RI
		M_1	M_2	M_3	
With S_9-Mix					
0	0.40 ± 0.18	26	73	2	1.76
50	0.59 ± 0.21^b	12	83	5	1.93
100	0.67 ± 0.22^b	3	97	–	1.97
Without S_9-Mix					
0	0.34 ± 0.12	66	34	–	1.34
50	0.40 ± 0.12	71	29	–	1.29
100	0.50 ± 0.24^a	57	43	–	1.43

SCE, sister chromatid exchanges; CPK, cellular proliferation kinetics; M_1, first mitotic division; M_2, second mitotic division; M_3, third or subsequent mitotic division; RI, replication index.
[a] ANOVA Significant differences compared to control and all doses ($P < 0.05$).
[b] *t*-test ($P < 0.01$).

the other hand, when CHO cells grew in the presence of the S9-Mix, SCE frequency showed a significant increase in both assayed concentrations ($P < 0.01$) (Table 2). In both experimental conditions the proportions of M_1, M_2 and M_3 cells and RI were not significantly modified in regard to control values ($P > 0.05$) (Table 2).

No deleterious effect was observed in human lymphocyte cultures treated with the TBZ formulation as revealed by the lack of increased frequency of SCEs ($P > 0.05$) (Fig. 1). On the contrary, a significant decrease of the MI ($P < 0.003$) and RI ($P < 0.05$) was observed (Table 3).

In CHO-K1 cells only a concentration of 200 $\mu g\ ml^{-1}$ of TBZ formulation induced a significant decrease of cell viability ($P = 0.05$), whereas no effect was observed within the 50–100 $\mu g\ ml^{-1}$ dose range (Table 4). A dose-dependent decrease in the MI of CHO-K1 was observed when treated with 50–200 $\mu g\ ml^{-1}$ of TBZ formulation, although it did not reach statistical significance ($P > 0.05$). The mitotic activity decreased in TBZ formulation-treated cultures with a mean *f* of 0.88, 0.63 and 0.29, for doses of 50, 100 and 200 $\mu g\ ml^{-1}$ of TBZ formulation, respectively (Table 4). In addition no differences in cell viability, MI or frequencies and types of spindle anomalies were observed in the negative controls (untreated and solvent-treated cells) from CHO cells, pooled data are presented for the control cultures.

Control CHO cells showed a mean frequency of 6.23% ± 0.65% of aberrant spindles including: multipolar (1.69% ± 0.01%) and degenerated figures (4.54% ± 0.65%). A significant increase of the aberrant mitotic spindles was observed related to both an enhanced frequency of multipolar and degenerated figures after treatment with 200 $\mu g\ ml^{-1}$ of TBZ formulation ($P = 0.01$). Although the level was not statistically significant, the
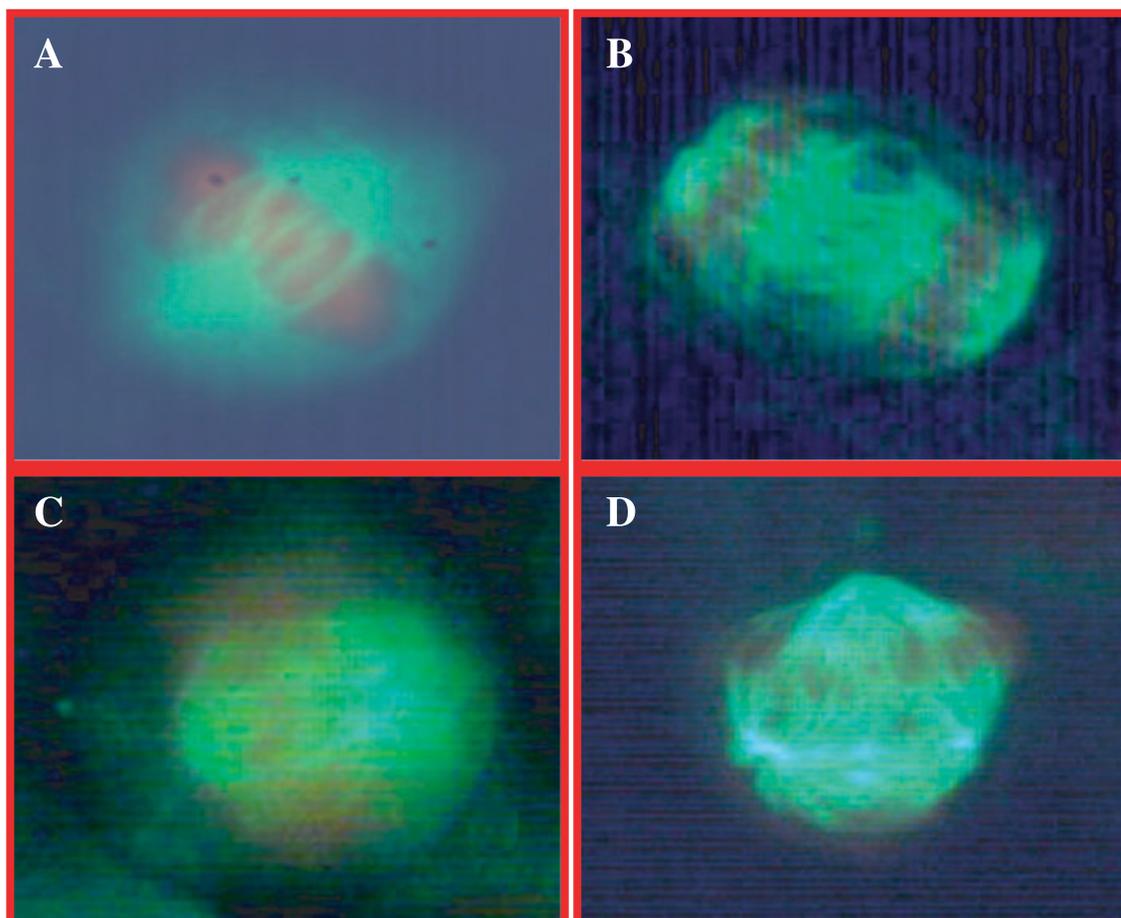
Figure 1. (A) A normal CHO cell metaphase. (B) A normal CHO cell anaphase. (C) Aberrant bipolar metaphase. (D) Aberrant multioolar metaphase (tripolar). This figure is available in colour online at www.interscience.wiley.com/journal/jat

Table 3. *In vitro* evaluation of sister chromatid exchanges (SCE), mitotic index (MI) and replication index (RI) in human peripheral blood cultures exposed to TBZ

Ind	TBZ concentration								
	SCE/cell ± SE	0 µg ml^{-1} RI	MI (%)	SCE/cell ± SE	50 µg ml^{-1} RI	MI (%)	SCE/cell ± SE	100 µg ml^{-1} RI	MI (%)
1	7.2 ± 0.4	2.3	5.8	7.5 ± 0.4	2.0	4.6	6.8 ± 0.4	1.7	3.6
2	8.3 ± 0.2	2.2	4.2	7.0 ± 0.3	2.2	3.8	5.2 ± 0.5	1.9	3.0
3	7.6 ± 0.6	2.4	4.7	8.1 ± 0.5	2.2	4.0	7.3 ± 0.3	1.7	3.3
4	6.1 ± 0.3	2.4	3.9	7.6 ± 0.4	2.4	3.5	8.1 ± 0.4	1.5	3.0
5	6.5 ± 0.5	2.3	5.2	6.5 ± 0.3	2.2	4.3	6.0 ± 0.3	1.9	3.7
6	5.9 ± 0.4	2.3	5.9	6.0 ± 0.4	2.2	5.0	6.0 ± 0.5	1.8	4.2
7	2.8 ± 0.7	1.3	5.5	7.2 ± 0.2	1.9	6.9	5.2 ± 0.3	1.23	1.4
8	4.6 ± 0.6	1.4	4.9	5.2 ± 0.1	1.8	3.1	7.3 ± 0.2	1.6	1.9
9	4.5 ± 0.3	1.4	3.3	4.8 ± 0.4	1.4	3.3	4.6 ± 0.4	1.18	1.7
10	7.0 ± 0.6	2.3	4.9	2.9 ± 0.5	1.7	5.6	3.7 ± 0.5	1.38	2.4
X ± SE	6.05 ± 0.53	2.03 ± 0.15	4.59 ± 0.27	6.28 ± 0.50	1.99 ± 0.10	4.41 ± 0.37	6.02 ± 0.43	1.59 ± 0.08[a]	2.82 ± 029[b]

Ind, human healthy blood donors; SCE, sister chromatid exchanges; RI, replication index.
[a] *t*-test ($P < 0.05$).
[b] ANOVA, significant differences compare with control ($P < 0.003$) and 50 µg ml^{-1} TBZ treatment ($P < 0.02$).

frequency of degenerated spindles was higher than that of multipolar spindles ($P > 0.05$) also in those cultures treated with 100 and/or 200 µg ml^{-1} of TBZ formulation, respectively (Table 4).

Discussion

Since previous studies with TBZ showed an effect on microtubule depolymerization, TBZ (or its metabolite)

Table 4. Mitotic spindles, cellular viability (CV), mitotic indexes (MI) and mitotic index factors (*f*) in control and TB-treated CHO cells after 24 h of treatment

TBZ concentration (µg ml^{−1})	CV	MI	*f*	Frequency (%) mitotic spindles			
				Normal	Aberrant	Multipolar	Degenerated
0	99.60 ± 0.29	3.43 ± 0.67	1.00	93.77 ± 0.65	6.23 ± 0.65	1.69 ± 0.01	4.54 ± 0.65
50	99.50 ± 0.50	3.05 ± 0.29	0.88	94.31 ± 0.73	5.69 ± 0.73	2.15 ± 0.38	3.53 ± 0.35
100	99.25 ± 0.06	2.19 ± 0.64	0.63	85.68 ± 1.50	14.32 ± 1.50	3.62 ± 0.34	10.86 ± 1.28
200	81.31 ± 1.12[a]	1.01 ± 0.07	0.29	56.37 ± 0.46	43.63 ± 0.46[b]	18.59 ± 1.68[b]	25.04 ± 1.64[b]

Results are presented as mean values of pooled data from three independent experiments ± standard error of the mean.
[a] $P \leq 0.05$; [b] $P \leq 0.01$.

seems to exert effects on cells which could explain the contradictory results obtained in different reports (Davidse and Flach, 1978; Mailhes *et al*., 1997). *In vitro* systems showed microtubule depolymerization (Wallin *et al*., 1988). The primary action of TBZ is to inhibit microtubule polymerization by binding to **β**-tubuline (Prichard, 1994). Several aneuploidy-inducing chemicals causing disruption of the spindle apparatus through inhibition of microtubule polymerization can lead to cell transformation with specific numerical chromosome aberrations taking an important role in the development of tumoral proliferation (Voutsinas *et al*., 1997).

Disassembly of microtubules is induced differentially by different chemicals depending on their specificity. Natarajan *et al*. (1993) described perturbations in the mitotic apparatus and spindle disturbances in pulmonary-derived Chinese hamster cell lines exposed to TBZ and Pisano *et al*. (2000) suggested an effect that disturbs the normal arrangement of microtubules. The development of an appropriate testing system for identifying aneugenic substances has become an important target in genetic toxicology. Statements about aneuploidy-inducing substances in mammalian cells are based on the results of cytogenetic investigations, such as metaphase chromosome counting and micronucleus assay with kinetochore staining.

The present results show differences in the cell survival and frequency of dividing cells after exposure to the TBZ formulation depending on the cell type being studied. The ability of TBZ formulation to induce spindle disturbances is not documented. The present findings reveal differences in the spontaneous frequency of aberrant spindles in transformed cells in agreement with previous results observed with the carbamate pesticide zineb as well as its commercial formulation azzurro, which is employed for eradication of fungal infection on fruit plants and vegetables among other properties (Soloneski *et al*., 2003). Several concepts give a framework to a new interpretation of TBZ action. The selective toxicity derives from the fact that specific, high-affinity binding to parasite *β*-tubuline occurs at much lower concentrations than the doses binding to the mammalian protein (Goodman and Gilman, 2001).

The generation of SCE has been related to errors in DNA replication (Tucker *et al*., 1993). Our studies suggest that TBZ by-products (metabolites) induce SCEs. This parameter considered as an endpoint has been employed commonly to evaluate cytogenetic responses to chemical exposure. No SCE changes occurred in *in vitro* human and CHO cells without S9-Mix enhancing the role of metabolic activation in potential indirect mutagens.

TBZ failed to accumulate mitotic cells in human lymphocyte cultures when compared with other benzimidazole derivatives (Holden *et al*., 1980) and prolonged the average cell generation time (Adler, 1993). Peripheral blood human cultures exhibited a sig-nificant decrease ($P < 0.003$ and $P < 0.05$) at the 100 µg ml^{−1} TBZ dose in the MI and RI which are considered as two informative biomarkers in agreement with Herrera *et al*. (1991). These authors described both markers as good genotoxic endpoints related to cytotoxicity and cellular proliferation kinetics. Genotoxicity of TBZ or its metabolites seems to be implicated in CPK modifications, as previously suggested (Mudry *et al*., 1986; 1987).

These new observations confirm that TBZ needs to be activated to induce genetic damage. Different studies demonstrated modifications of cytochrome P450 enzymes (*CYP*) by imidazolic compounds (Simon *et al*., 1991). In terms of pharmacokinetics, imidazoles such as TBZ are metabolized by *CYP* families (Guenguerich, 1987), being potent inductors of the *CYP1A1* and *CYP1A2* families which have been detected in mammals (Mori *et al*., 1993). These findings agree with mouse Comet assay results (Sasaki *et al*., 1997) in which TBZ induced DNA damage in all the organs studied in this short term test, supporting the *in vivo* DNA-damaging action.

The importance of the selected endpoints and the tests that represent them for TBZ and/or their metabolites, enhance their value in characterizing potential mutagenicity. In fact, the expression of mutagenic activity *per se* (the ability of a chemical to produce alterations in DNA structure content) is clearly a critical mechanistic consideration when assessing suspected genotoxic activity.

References

Adler ID. 1993. Synopsis of the *in vivo* test results obtained with the 10 known or suspected aneugens tested in the CEC collaborative study. *Mutat. Res.* **287**: 131–137.

Adler ID, Kliesch U, Van Hummelen P, Kirschvolders M. 1991. Mouse micronucleus tests with known and suspect spindle poisons: results from two laboratories. *Mutagenesis* **6**: 47–53.

Carballo M, Mudry MD, Larripa IB, Villamil E, D'Aquino M. 1992. Genotoxic action of an aqueous extract of *Heliotropium curassavicum var. Argentium*. *Mutat. Res.* **279**: 245–253.

Davidse LC, Flach W. 1978. Interaction of thiabendazole with fungal tubulin. *Biochem. Biophys. Acta* **543**: 82–90.

Davies HD, Sakuls P, Keystone JS. 1993. Creeping eruption. A review of clinical presentation and management of 60 cases presenting to a tropical disease unit. *Arch. Dermatol.* **129**: 588–591.

Delescluse C, Ledirac N, Li R, Piechocki MP, Hines RN, Gidrol X, Rahmani R. 2001. Induction of cytochrome P450 1A1 gene expression, oxidative stress and genotoxicity by carbaryl and thiabendazole in transfected human HepG2 and lymphoblastoid cells. *Biochem Pharmacol* **61**(4): 399–407.

Garner RC, Miller EC, Miller JA. 1972. Liver microsomal metabolism of aflatoxin B 1 to a reactive derivative toxic to *Salmonella typhimurium* TA 1530. *Cancer Res.* **32**: 2058–2066.

Guenguerich FP. 1987. *Mammalian Cytochromes P 450*, vol. 1. CRC Press: Boca Raton, FL.

Guglielmi GE, Vogt TF, Tice RR. 1982. Induction of sister chromatid exchanges and inhibition of cellular proliferation *in vitro*, I. Caffeine. *Environ. Mutagen.* **4**: 191–200.

Herrera LA, Tittelbach E, Gebhart E, Ostrosky-Wegman P. 1991. Change in the proliferation of human lymphocytes induced by several cytostatics and revealed by premature chromosome condensation technique. *Mutat. Res.* **263**: 101–106.

Holden HE, Crider PA, Wahrenburg MG. 1980. Mitotic arrest by benzimidazole analogs in human lymphocyte cultures. *Environ. Mol. Mutagen.* **2**: 67–73.

Kappas A, Georgopoulos SG, Hastie AC. 1974. On the genetic activity of benzimidazole and thiophanate fungicides on diploid. *Aspergillus nidulans. Mutat Res.* **26**: 17–27.

Kochendörfer U, Stammberger I, Mayer D, Schwanitz G. 1996. A new possible parameter for the detection of aneuploidy inducing substances: the analysis of qualitative and quantitative abnormalities of the spindle apparatus. *Mutat. Res.* **361**: 55–66.

Leopardi P, Zijno A, Bassani B, Pacchierotti F. 1993. *In vivo* studies on chemically induced aneuploidy in mouse somatic and germinal cells. *Mutat Res.* **287**(1): 119–130.

Mailhes JB, Young D, Aardema MJ, London SN. 1997. Thiabendazole-induced cytogenetic abnormalities in mouse oocytes. *Environ. Mol. Mutagen.* **29**: 367–371.

Matsushima T, Sawamura M, Hara K, Sugimura T. 1976. A safe substitute for polychlorinated biphenyls as an inducer of metabolic activation system. In: Serres FJ, Fouts JR, Bend JR, Philpot RM (Eds.), *In vitro* metabolic activation in mutagenesis testing, Elsevier/North-Holland, Amsterdam, 85–88.

McGahon AJ, Martín SJ, Bissonnette RP, Mahboubi A, Shi Y, Mogil RJ, Nishioka WK, Green DK. 1995. The end of the (cell) line: methods for the study of apoptosis *in vitro*. *Methods Cell. Biol.* **46**: 153–185.

Migliore L, Neri M. 1991. Evaluation of twelve potential aneuploidogenic chemicals by the *in vitro* human lymphocyte micronucleus assay. *Toxicol. in Vitro* **5**: 325–336.

Miller BM, Adler ID. 1989. Suspect spindle poisons: analysis of c-mitotic effects in mouse bone. *Mutagenesis* **4**: 208–221.

Miller B, Adler ID. 1992. Aneuploidy induction in mouse spermatocytes. *Mutagenesis* **7**: 69–76.

Mori Y, Limura K, Hirano K. 1993. N-Benzymidazole, a potent inducer of rat liver enzymes involved in mutagenic activation of various carcinogens. *Mutat. Res.* **302**: 129–133.

Mudry MD, Gadano A, González M, Carballo MA. 1995. Mutagénesis Química: riesgo y beneficio en el consumo de Antiparasitarios. (Review). *Interciencia* **20**: 204–211.

Mudry de Pargament MD, Labal de Vinuesa M, Larripa I. 1986. Evaluación citogenética del daño inducido por benzimidazoles de uso terapéutico en parasitología. *Medicina* **46**: 125–126.

Mudry de Pargament MD, Labal de Vinuesa M, Larripa I. 1987. Mutagenic bioassay of certain pharmacological drugs I. Thiabendazole (TBZ). *Mutat. Res.* **188**: 1–6.

Nakagawa Y, Moore GA. 1995. Cytotoxic effects of postharvest fungicides, ortho-phenylphenol, thiabendazole and imazalil, on isolated rat hepatocytes. *Life Sci.* **57**: 1433–1440.

Natarajan AT. 1993. An overview of the results of testing of known or suspected aneugens using mammalian cells *in vitro*. *Mutat. Res.* **287**: 113–118.

Natarajan AT, Duivenvoorden WC, Meijers M, Zwanenburg TS. 1993. Induction of mitotic aneuploidy using Chinese hamster primary embryonic cells. Test results of 10 chemicals. *Mutat. Res.* **287**: 47–56.

Parry JM, Sors A. 1993. The detection and assessment of the aneugenic potential of environmental chemicals: the European Community Aneuploidy Project, *Mutat. Res.* **287**: 3–15.

Perry P, Wolff S. 1974. New Giemsa method for differential staining of sister chromatids. *Nature (London)* **261**: 156–161.

Pisano C, Battistoni A, Antoccia A, Degrassi F, Tanzarella C. 2000. Changes in the organization after exposure to a benzimidazole derivative in Chinese hamster cells. *Mutagenesis* **15**: 507–515.

Prichard R. 1994. Antihelminthic resistance. *Vet. Parasitol.* **54**: 259–268.

Ramirez, Vanessa, Cuenca, Patricia. 2000. Micronuclei frequency in lymphocytes of individuals occupationally exposed to pesticides. *Rev. Biol. Trop.* **49**(1): 1–8.

Reygrobellet C, Eeckhoutte C, Sutra JF, Alvinerie M, Galtier P. 1996. Major involvement of rabbit liver cytochrome P450 1A in thiabendazole 5-hydroxylation. *Xenobiotica* **26**: 765–778.

Sasaki YF, Saga A, Akasaka M, Nishidate E, Su YQ, Matsusaka N, Tsuda S. 1997. *In vivo* genotoxicity of orthopenylph-enol, biphenyl, and thiabendazole detected in multiple organs by the alkaline single cell electrophoresis assay. *Mutat. Res.* **395**: 189–198.

Silverstein RA, Richardson W, Levin H, Allshire R, Ekwall K. 2003. A new role for the transcriptional corepressor SIN3; regulation of centromeres. *Curr Biol.* **13**(1): 68–72.

Simon WA, Büdigen C, Fahr S, Kinder B, Koske M. 1991. The H+K+-ATPase inhibitor pantoprazole (BY1023/SK&F96022) interacts less with cytochrome P450 than omeprazole and lanzoprazole. *Biochem. Pharmacol.* **42**: 347–355.

Soloneski S, Reigosa MA, Larramendy ML. 2003. Effect of the dithiocarbamate pesticide zineb and its commercial formulation, the azzurro. V. Analysis of qualitative and quantitative abnormalities induced in the spindle apparatus of transformed and non-transformed cell lines. *Mutat. Res.* **536**: 121–129.

Styles JA, Garner R. 1973. Benzimidazolecarbamate methyl ester evaluation of its effects *in vivo* and *in vitro*. *Mutat. Res.* **26**: 177–187.

Tracy JW, Webster T, Jr. 2001. Drugs used in the chemotherapy of parasitic infections. In Goodman, Gilman's (consult eds.), Hardman JG, Limbird LE (eds.). The pharmacological basis of therapeutics, 10th int edn. McGraw-Hill, New York, pp 1059–1140.

Tucker JD, Auletta A, Cimino MC, Dearfield KL, Jacobson-Kram D, Tice RR, Carrano AV. 1993. Sister-chromatid exchange: Second report of the Gene-Tox program. *Mutat. Res.* **297**: 101–180.

Van Hummelen P, Elhajouji A, Kirsch-Volders M. 1995. Clastogenic and aneugenic effects of three benzimidazole derivatives in the *in vitro* micronucleus test using human lymphocytes. *Mutagenesis* **10**: 23–29.

Voutsinas G, Zarani FE, Kappas A. 1997. The effect of environmental aneuploidy-inducing agents on the microtubule architecture of mitotic meristematic root cells in *Hordeum vulgare*. *Cell Biol. Int.* **21**: 411–418.

Wallin I, Friden B, Billger M. 1988. Studies on the interaction of chemicals with microtubule assembly *in vitro* which can be used

as an assay for the detection of cytotoxic chemicals and possible inducers of aneuploidy. *Mutat. Res.* **201**: 303–311.

Warr TJ, Parry EM, Parry, JM. 1993. A comparison of two *in vitro* mammalian cell cytogenetic assays for the detection of mitotic aneuploidy using 10 known or suspected aneugens. *Mutat. Res.* **287**: 29–46.

Watanabe-Akanuma M, Ohta T, Sasaki YF. 2005. A novel genotoxic aspect of thiabendazole as a photomutagen in bacteria and cultured human cells. *Toxicol. Lett.* **158**: 213–219.

Zar J. 1984. *Biostatistical Analysis*. Prentice-Hall, Inc. New York 201–292.