International Journal of Molecular Medicine and Advance Sciences 6 (4): 54-58, 2010
ISSN: 1813-176X

Clastogenic Studies on Hexaconazole: A Triazole Fungicide in Rats

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Abstract: Hexaconazole is a triazole fungicide used in crop protection. Triazoles are one of the promising groups of fungicides that act by inhibiting the biosynthesis of ergosterol, an essential component of fungal cell membrane, via inhibition of cytochrome P450 dependent enzyme lanosterol 14α-demethylase. The present study is aimed to screen hexaconazole for its *in vivo* clastogenic potential in Wistar strain rats. The doses of hexaconazole selected in the present study were 182, 365 and 730 mg kg^{-1} for male rats and 506, 1012 and 2024 mg kg^{-1} for females. These doses were corresponding to 1/12th, 1/6th and 1/3rd, respectively of earlier reported oral LD50 values which were 2189 mg kg^{-1} in males and 6071 mg kg^{-1} in females. The *in vivo* clastogenic potential of hexaconazole was studied employing bone marrow chromosomal aberrations assay and micronucleus test following single exposure and multiple exposures to the drug. Results of bone marrow chromosomal aberrations assay indicated that hexaconazole in the tested doses is incapable of producing any structural or numerical aberrations in both male and female rats. Analysis of the bone marrow smears of the slides for micronucleated polychromatic erythrocytes revealed no significant increase in their number in hexaconazole treated rats. Multiple exposures also could not enhance their incidence significantly. These observations confirmed the results of chromosomal aberrations assay in this study where hexaconazole failed to produce and any aberrations. It further appears from the present study that hexaconazole had no effect on spindle formation during cell division.

Key words: Triazole, hexaconazole, clastogenic effect, bone marrow chromosomal aberrations, micronucleus test, India

INTRODUCTION

Hexaconazole is a triazole fungicide used in crop protection. Triazoles are one of the promising groups of fungicides. They are broad spectrum systemic fungicides that offer both protective and curative effects (Bohmont, 1996). Triazoles inhibit the biosynthesis of ergosterol, an essential component of fungal cell membrane, via inhibition of cytochrome P450 dependent enzyme lanosterol 14α-demethylase (Van den Bossche *et al*., 1986). Depletion of ergosterol in the fungal cell membrane results in altered membrane fluidity thereby reducing the activity of membrane associated enzymes. This leads to increased permeability and subsequent inhibition of cell growth and replication (Como and Dismukes, 1994). In addition, azoles may exhibit other direct effects on cell membrane fatty acids and can inhibit cytochrome P 450 dependent enzymes of fungal respiration chain (Uno *et al*., 1982).

Among the potential ill effects of pesticides, mutagenicity, clastogenicity and carcinogenicity are especially significant. Any agent that causes chromosome aberrations is known as clastogen (Hoffman, 1996). Genetic alterations of a clastogenic type have been reported to be associated with a variety of human cancer and birth defects (Radman *et al*., 1982; Brusick, 1987). In order to protect the health and genetic heredity of humans and animals, the genotoxic potential of various xenobiotics used by mankind has to be studied. Thus the present study is aimed to screen hexaconazole for its *in vivo* clastogenic potential in rats.

MATERIALS AND METHODS

Wistar strain rats of either sex, aged between 10-12 weeks, hexaconazole (Technical grade 92.5%, M/s Rallis India Limited, Agro Chemical Division, Mumbai-400 703), dissolved in dimethylsulphoxide (DMSO) (S.d. Fine-Chem Ltd., Mumbai) and cyclophosphamide (Sigma, USA) dissolved in distilled water were used in the study. The *in vivo* clastogenic potential of hexaconazole was studied employing bone marrow chromosomal aberrations assay and micronucleus test.

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Table 1: Grouping and treatment of animals for bone marrow chromosomal aberrations assay study on hexaconazole

Groups	No. of animals*	Treatments
I (Negative control/solvent control)	12	Dimethylsulphoxide (Both sexes: 1 mL kg^{-1})
II (Hexaconazole-low dose)	12	Hexaconazole (Male: 182 mg kg^{-1}; female: 506 mg kg^{-1})
III (Hexaconazole-medium dose)	12	Hexaconazole (Male: 365 mg kg^{-1}; female: 1012 mg kg^{-1})
IV (Hexaconazole-high dose)	12	Hexaconazole (Male: 730 mg kg^{-1}; female: 2024 mg kg^{-1})
V (Positive control)	12	Cyclophosphamide (Both sexes: 25 mg kg^{-1})

*Six animals in each group were sacrificed 24 h after first exposure

Bone marrow chromosomal aberrations assay: Sixty rats comprising of 30 males and 30 females were randomly divided into five groups, each consisting of 12 rats of either sexes in equal number. Out of the twelve rats in each group, six rats were treated orally, once with single dose of hexaconazole where as the remaining six rats were subjected to five oral exposures on 5 consecutive days. The animals were sacrificed 24 h after the last dose in either case. The doses of hexaconazole selected in the present study were 182, 365 and 730 mg kg^{-1} for male rats and 506, 1012 and 2024 mg kg^{-1} for females. These doses were corresponding to 1/12th, 1/6th and 1/3rd, respectively of earlier reported (Tomlin, 1995) oral LD50 values which were 2189 mg kg^{-1} in males and 6071 mg kg^{-1} in females. The drug was dissolved in DMSO. Cyclophosphamide, dissolved in distilled water was given at 25 mg kg^{-1} body weight which served as positive control. The grouping of animals was as shown in Table 1.

Harvesting of bone marrow: Rats were administered with colchicine (Sigma, USA) at 2 mg kg^{-1} body weight IP 2 h before sacrificing under ether anesthesia. The bone marrows were harvested as described by Preston *et al.* (1987). Both femurs were quickly removed from sacrificed animals and were placed on the edge of a plastic centrifuge tube. Both ends of femurs were crushed with forceps in the inner lip of the tube and the fragments were washed down into the test tube with little quantity of Normal Saline (NS) pre warmed to 37°C. Now bone marrow cells from the shaft of each femur were flushed thoroughly with NS using syringe and needle. Final volume of the suspension in the tube was made to 10 mL. To ensure free suspension of marrow cells in NS, the test tubes were placed on a cyclomixer for about a minute. The test tubes were then left undisturbed for 3 min to allow the fragments to settle to the bottom. The suspension was then decanted to another tube and centrifuged for about 4 min near 800 rpm. The supernatant was slowly removed by gentle aspiration, leaving a small volume over the pellet. Then the pellet was resuspended in the remaining volume.

Hypotonic treatment: About 5 mL of freshly prepared 0.075 M potassium chloride was added to the resuspended pellet and the tubes were incubated for 20 min at 37°C in a water bath.

Fixation: About 0.5 mL of freshly prepared fixative made of methanol and glacial acetic acid in the ratio of 3:1 was added to each tube. The tubes were allowed to stand at room temperature for 15-20 min and were centrifuged at 800 rpm for 4 min to obtain the pellet. The supernatant was aspirated out and the pellet was resuspended in the remaining small volume of solution. About 2.0 mL of fixative was added to the tubes, centrifuged at 800 rpm for 4 min and pellet obtained. This step was repeated twice and resuspended pellet was finally made upto a volume of 0.5 mL with fixative.

Slide preparation: Two drops of the suspension were dropped onto clean chilled wet slide placed at an angle and the slides were dried over slide warmer. The slides were then stained in Giemsa (1:40) for 3-4 min.

Screening for aberrant cells: The slides were coded and scored for chromosomal aberrations. About 100 metaphase spreads were analyzed for each animal. The aberrations in hexaconazole treated groups were compared with concurrent negative controls.

The micronucleus test: Treatment and grouping of animals for the micronucleus test were similar to those described in the bone marrow chromosomal aberrations assay.

Extraction of bone marrow and preparation of slides: The bone marrow slides were prepared as per the procedure of Schmid (1975). Both the femurs were removed and the both the ends of each femur were chipped off with forceps. The marrows were thoroughly flushed into clean test tubes using syringe and needle that was pre loaded with 5 mL of fetal calf serum (Hi-media). The cells were then suspended by vigorous agitation of the tubes on a cyclomixer for 30 sec. After centrifugation at 800 rpm for 5 min, the supernatant serum was discarded and the pellet was resuspended in small volume of fresh serum. A drop of this suspension was smeared onto a clean glass slide and air dried. The slides were then immediately fixed in methanol for 10 min and stained in May-Gruenwald stain followed by Giemsa as described by Schmid (1975).

Evaluation of slides: One thousand Polychromatic Erythrocytes (PCEs) per animal were screened under oil immersion and the Micronucleated PCEs (MNPCEs) were counted.

Statistical analysis: The data were analyzed by χ^2-test as described by Snedecor and Cochran (1968).

RESULTS

Bone marrow chromosomal aberrations: One hundred spreads per animal were analyzed and the observations were expressed as percentage of aberrant cells in each group. The spreads were screened for breaks and other types of aberrations like centric fusion, centromeric attenuation, ring chromosome, end to end association and polyploidy. The results of single and multiple exposure studies are shown in Table 2 and 3, respectively and they indicated that hexaconazole did not induce chromosomal aberrations at any dose level studied after both single and multiple exposures. However, positive control, cyclophosphamide, produced chromosomal aberrations in both the studies.

Table 2: Effect of hexaconazole (single exposure) on chromosomal aberrations in bone marrow cells of rat

Groups	No. of spreads analyzed	No. of aberrations		No. of aberrant cells	Aberrant cells (%)
		Breaks	Others		
I (Negative control)	600	1	0	1	0.17
II (Low dose)	600	2	0	2	0.33
III (Medium dose)	600	1	0	1	0.17
IV (High dose)	600	3	0	3	0.50
V (Positive control)	600	69	0	69	11.50**

** p<0.01

Table 3: Effect of hexaconazole (multiple exposures) on chromosomal aberrations in bone marrow cells of rat

Groups	No.of spreads analyzed	No. of aberrations		No. of aberrant cells	Aberrant cells (%)
		Breaks	Others		
I (Negative control)	600	2	0	2	0.33
II (Low dose)	600	1	0	1	0.17
III (Medium dose)	600	4	0	4	0.67
IV (High dose)	600	3	0	3	0.50
V (Positive control)	600	76	2	78	13.00**

**p<0.01

Table 4: Effect of hexaconazole (single exposure) on the frequency of micronuclei in bone marrow cells of rat

Groups	No. of PCEs scored	No. of MNPCEs	No. of MNPCEs/10^3 PCEs
I (Negative control)	6000	9	1.50
II (Low dose)	6000	11	1.83
III (Medium dose)	6000	10	1.67
IV (High dose)	6000	13	2.16
V (Positive control)	6000	53	8.83**

**p<0.01

Table 5: Effect of hexaconazole (multiple exposures) on the frequency of micronuclei in bone marrow cells of rat

Groups	No. of PCEs scored	No. of MNPCEs	No. of MNPCEs/10^3 PCEs
I (Negative control)	6000	11	1.83
II (Low dose)	6000	10	1.67
III (Medium dose)	6000	8	1.33
IV (High dose)	6000	16	2.67
V (Positive control)	6000	62	10.33**

**p<0.01

Bone marrow micronuclei: The results were expressed as number of micronucleated polychromatic erythrocytes per thousand polychromatic erythrocytes (MNPCEs/10^3PCEs) (Table 4 and 5). It was obvious from the results that the exposure of rats to either single dose or to multiple doses of hexaconazole for 5 days did not produce any significant increase in the incidence of micronuclei. As expected the positive control drug cyclophosphamide significantly increased the occurrence of micronuclei.

DISCUSSION

Results of bone marrow chromosomal aberrations assay indicated that hexaconazole in the tested doses is incapable of producing any structural or numerical aberrations in both male and female rats. To ensure correctness of the procedure followed, a known clastogen, cyclophosphamide was used as positive control at recommended dose level as suggested by Preston *et al.* (1987) and Oldham (1997). Although, a single exposure would in the majority of cases provide for maximum sensitivity of assay (Preston *et al.*, 1987), multiple exposures were also followed in the present study to ensure adequate concentration of the drug in bone marrow.

Analysis of the bone marrow smears of the slides for micronucleated polychromatic erythrocytes revealed no significant increase in their number in hexaconazole treated rats. Multiple exposures also could not enhance their incidence significantly. These observations confirmed the results of chromosomal aberrations assay in this study where hexaconazole failed to produce and any aberrations. It further appears from the present study that hexaconazole had no effect on spindle formation during cell division.

The micronucleus test, *in vivo* is a method devised primarily for screening chemicals for chromosome breakage. The test is as sensitive as metaphase method; in addition it also detects the effects on the spindle apparatus (Schmid, 1975). In anaphase, acentric chromatid and chromosome fragments lag behind when the centric elements move towards the spindle poles. After telophase the undamaged chromosomes as well as the centric fragments give rise to regular daughter nuclei. The lagging elements are also included in the daughter cells but a considerable portion is transformed into one or

several secondary nuclei which are as a rule much smaller than principal nucleus and are therefore called micronuclei. These micronuclei still remain in the immature erythrocytes (polychromatic erythrocytes) even after the extraction of the regular nucleus (Schmid, 1975). Thus an increase in the frequency of micronucleated polychromatic erythrocytes indicates that the administered compound had interfered with nuclear division of the bone marrow erythrocytes in such a way that chromatin fragments or whole chromosome had lagged at anaphase and failed to be incorporated into one of the daughter nuclei at the time of the cell division.

The negative results obtained in chromosomal aberrations assay and micronucleus test are in accordance with the earlier reports on some triazole compounds. Amitrole, a triazole herbicide did not increase mutation frequencies in test systems involving two genera of bacteria, drosophila and human lymphocyte cell cultures (Sorsa and Gripenberg, 1976; Laamanen *et al*., 1976; Meretoza *et al*., 1976). WHO (1974) also reported negative results in salmonella strains and yeast cells with amitrole. Mattioli *et al*. (1994) also reported no evidence of DNA fragmentation in *in vitro* studies in rats with amitrole. Another triazole, triadimefon neither increased the frequency of micronucleated polychromatic erythrocytes in bore marrow cells of mice nor had any sister chromatid exchange inducing effect in human lymphocyte cell culture (Kevekordes *et al*., 1996).

CONCLUSION

Triazoles are often implicated in thyroid carcinogenesis and carcinogenesis is often related to mutagenecity. The 3-amine-1, 2, 4-triazole was associated with thyroid tumours in rats (Hiasa *et al*., 1982; Tsuda, 1975; Steinhoff *et al*., 1983; Mattioli *et al*., 1994). Diniconazole was also reported to induce thyroid tumors in rats (Hosokawa *et al*., 1993). However, none of the studies established the genotoxic effect of triazoles behind thyroid tumor induction and rather a hormonal disregulation mechanism was suggested for the said tumerogenic effect (Hosokawa *et al*., 1993; Mattioli *et al*., 1994).

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