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Genotoxicity of Newly Synthesised Imidazole-2(2-2 benzylidene hydrazinyl)-5,5 diphenyl-1,5dihydro -4-imidazol-4one(4-chloro derivative)

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ABSTRACT

The newly synthesised 2-(2-2 benzylidene hydrazinyl)-5,5 diphenyl-1,5dihydro -4-imidazol-4one was found to have anthelmintic, ,antibacterial and antifungal activity . Since the Compound has the potential for further development .Genotoxicity of the new Compound was studied for visible changes to chromosome structure and morphology .They have played a very important part as indicators of genetic damage in both clinical and cancer studies.The Chromosome Aberration Assay Invitro is a useful and sensitive test for detection of Genotoxins .There are four common types of structural Chromosome Aberrations are

1.Deletions 2.Duplications 3.Inversions 4.Translocations.

Micronuclei are indicators for fixed genomic damage in cells .The Synthesised compound did not show any Chromosomal Aberrations or formation Micronuclei

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Key words: Genotoxicity, Mutagenecity, Chromosomal aberrations, Micro nuclei, Carcinogenecity.

INTRODUCTION

Mutations in DNA may lead to cancer that disrupts these orderly process by disrupting the programming regulating process .in cultured cells certain phenotypic alterations that are characteristic of tumerogenic cells will be inducted and the cell transforms and acquire the characteristics of malignant cells.

Most aberration inducing agents can produce lesions into chromatin at all stages of the cell cycle. (Cancer research 34, 2266-2273, sept1974)

Chromosome aberration assay in-vitro is a useful and sensitive test for detection of Geno toxins. The accumulation of genetic changes may lead to genetic instability which may result in cancer it has been demonstrated that Micronuclei frequencies besides chromosome aberration frequencies are a cytogenetic end point providing strong evidence for the association between the extent of chromosomal changes and cancer risk.

MATERIALS AND METHODS

Preparation of s9 mix

1. Mitomycin c stock solution : 0.05mg in 100mlof sterile water
2. Mitomycin c working solution : 10µl of stock solution to 50µl
3. Cyclophosphamide stock solution : 0.05mg in 100ml of sterile water.
4. Cyclophosphamide working solution: 10µl of stock solution to 50ml using Sterile water.
5. NADPH solution : 1.5mg in 5ml distilled water
6. 0.4M Magnesium Chloride and 0.65M Potassium Chloride solution
7. Glucose -6-Phosphate solution : 700mg in 5ml distilled water.

9 0.1M Dipotassium Hydrogen Phosphate : 175 g in 1000ml.

10.1M Potassium Dihydrogen Phosphate: 136g in 1000ml.

11.0.2M Phosphate Buffer solution: pH 7.4(80.2mlof K_2HPO4 and KH2PO4 were mixed and the PH was adjusted to 7.4 and was made to 500ml.
0.15 M Potassium Chloride solution:13.5g in 1.2L of water.
12.Normal Saline :27g in 3000ml
13.Geisma stain
14.Trypsene –Versene solution

Preparation of culture media

Powdered Dulbecco's modified medium with 10% foetal Calf Serum.
1X McCoy's 5a medium with Hepes buffer plus 15% Foetal Calf serum

Methodology procedure for cell culture

The CHO cells have been used to study the chromosomal aberration induced by the new compound in comparison with the Mitomycin and Cyclophosphamide.The CHO cells are fibroblastic in nature with 12-14 hour cell cycle time and at Mitosis the cells roundup and should be easy to dislodge by shaking.The 'Mitotic Shake Off Method' was followed to remove the culture cells .Monolayer cultures of CHO cells approximately 60-80% confluent were for the study .in the glass bottle of 25cm² 1,5x106 cells Produced, Control, Mitomycin, Cyclophosphamide and the Imidazole derivative. All the cultures were prepared in duplicate. The Monolayer were rinsed with about 10ml of pre-warmed Phosphate Buffer Saline (PBS) and the PBS was discarded.The cells were rinsed with 3-5ml of pre-warmed Trypsin –Versene solution and excess removed immediately. The cells were observed for signs of rounding up and detaching from the glass bottle (5-10min) then resuspended the cells in 10ml of McCoy's

5a medium with 15% Fetal Calf Serum

Procedure for chromosomal aberration studies

After the cells were exposed to the test materials the steps followed were

1. Colchicine solution of 0.2ml of a 10µg/ml was added two hours before harvesting.
2. At the end of the incubation period (1.5CELL CYCLES),each glass bottle was shook gently to dislodge mitotic cells and transfer the cell suspension to labeled 15ml centrifuge tube and centrifuged at low speed (600rpm) for 5 min.
3. The supernatant was removed and the cells were gently resuspended in the residual medium and add freshly prepared 5ml of 0.075M Potassium Chloride.
4. The contents were left for three min at room temperature ,then were centrifuged for 5minutes and the supernatant was discarded.
5. 5ml of freshly prepared Methanol : Acetic acid fixative (3:1) was added drop wise manner while shaking the tube gently to resuspend the cells.
6. The contents were centrifuged at a higher speed (1200 rpm) for 5 min and the supernatant was discarded.
7. The last two steps were repeated once again.Five ml of fresh fixative was added and the tubes allowed to stand for 30 minutes.
8. Then the contents were centrifuged ,supernatant was removed and the cell button was re-suspended in 3-4 drops of fixative.
9. Using a Pasteur Pipette 3-4 drops of the suspension was transferred onto a microscope slide, previously soaked in alcohol ,polished with lens tissue and cooled to 4°c from a few inches above the slide and were checked for the degree of spreading of the metaphases.
10. The slides were dried and stained with Geimsa.
11. Atleast four slides were prepared from each culture.
12. The slides were rinsed off the stain with phosphate buffer and were shaken thoroughly to remove buffer and then air dried.
13. The slides were dipped in Sulphur free Xylene for a few seconds, and few drops of mounting medium.
14. Then the cover slip was placed without air bubbles and left overnight Until mounting sets.
15. The slides were observed under 100X magnification with oil immersion using Carl Zeiss Axioplan microscope.
16. The plates were observed for any kind of aberrations in the chromosomes.

Procedure for micronucleus test

After the cells are exposed to test materials using the previous procedure (used in chromosomal aberration)

1. Cytochalasin-B solution of 0.2ml of a 24µg/ml was added sixhrs before harvesting.
2. At the end of the incubation period ,each glass bottle was shook gently to dislodge mitotic cells and transfer the cell suspension to labelled 15ml centrifuge tube and centrifuged at low speed (600rpm) for 5 min.
3. The supernatant was discarded. 5ml of freshly prepared Methanol: Acetic acid fixative ((3:1) was added drop wise manner while shaking the tube gently to resuspend the cells.
4. The contents were centrifuged at a higher speed (1200rpm) for 5 min and the supernatant was discarded.
5. Five ml of freshly prepared Methanol : Acetic acid fixative(3:1) was added drop-wise manner while shaking the tube gently to re suspend the cells.
6. The contents were centrifuged at a higher speed(1200 rpm) for five minutes and the supernatant was discarded.
7. Five ml of fresh fixative was added and the tubes were allowed to stand for 30 minutes.
8. Then the were centrifuged, supernatant was removed and the cell button was resuspended in 3-4 drops of fixative.
9. Using a Pasteur Pipette 3 to 4 drops of the suspension was transferred onto a microscope slide, previously soaked in alcohol, polished with lens tissue and cooled to 4 degree centigrade, from a few inches above the slide and to check the degree of spreading of the cells.
10. The slides were Air dried and stained with Giemsa.
11. Atleast four slides were prepared for each culture. Each slide was rinsed with 5ml geimsa in horizontal staining over a sink for 5 min.
12. The slides were rinsed off with Phosphate buffer, and were shaken thoroughly to remove buffer and then air dried.
13. The intensity of staining was checked before mounting.The slides were dipped in sulphur- free xylene for a few seconds , add then in a few drops of mounting medium(eg DPX).
14. The cover slip was placed after expelling the air bubbles.
15. The slides were left for over night until the mounting medium completely sets.
16. The slides were observed under 100X magnification with oil immersion using Carl Zeiss Axioplan microscope to observe the presence of Micronuclei.

RESULTS AND DISCUSSION

Positive control Mitomycin C without s9 fraction, showed aberrations like gaps , fragments, deletions and breakage showing that it is a mutagen.

Cyclophosphamide is mutagenic in the presence of s9 fraction. It produced gaps , fragments, deletions and breakages showing that it is a mutagen.

Test material did not produce any gaps, fragments ,deletions and breakages and no formation of micronuclei, showing that it is not mutagenic and not carcinogenic.

Table 1: Chromosomal aberrations

controls	Breaks in chromosomes	fragments	Trinucleate chromatids	Deletions
Mitomycin c	present	present	present	present
Cyclophosphamide with s9	Present	Present	Present	present
Test sample	absent	Absent	Absent	absent

Table 2: Micronuclei formation

Controls	Micronuclei	Inference
Mitomycin c	present	mutagenic
cyclophosphamide	present	mutagenic
Test sample	absent	Non-mutagenic

The above results show that

1. Chromosomal Aberrations are present in the form of breaks in Chromosomes, Fragments , Trinucleate Chromatids and Deletions in the slides of Mitomycin C and Cyclophosphamide.
2. But such Aberrations are not present in the slides of test sample in concentration of 100mg/ml.
3. Mirco Nuclei are seen in the slides of Mitomycin C and Cyclophosphamide .but MicroNuclei are not present in the slides of test sample in concentration of 100mg/ml.

CONCLUSION

The compound has Anthelmintic, Antibacterial, Antifungal activity after the study it is found that it has no Mutagenicity and Carcinogenicity .Further activity study can be done on this Compound.

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