3.1 Test Animals

The mouse is the model organism most closely related to humans. The mouse and human genomes are approximately the same size, contain the same number of genes and show extensive conserved gene order. Most human genes have mouse counterparts and the functions of these genes are closely related. Mutations that cause diseases in humans often cause similar diseases in mice. Importantly, mice have genes that are not represented in other animal models (the fruit fly and nematode worm), including the genes of the immune system.

The mouse has further properties that make it an ideal model organism. Mice are small, easy to maintain in the laboratory and (compared to most mammals) have a short breeding cycle (about 2 months). They can produce 10-15 offspring per litter and approximately one litter every month. This makes them suitable for genetic analysis. Many mutants are available and new mutations can be introduced easily by irradiation, feeding with chemical mutagens or inserting DNA fragments into the genome to interrupt genes. The work has proper approval of institutional animal ethics committee.

Swiss albino mice, comprising of both the sexes, in the age group of 10 – 12 weeks and weighing 20 – 25g obtained from Pasture Institute, Shillong, India, were used as the mammalian in vivo test system in the present study. The animals were maintained in laboratory conditions at a room temperature of 25+5° C and in 12 h dark and 12 h light cycles usually four to five animals were housed in a single polypropylene cage for breeding. Animals receiving various treatments were housed individually or in treatment groups. The individuals were identified by cage cards. Food
(standard food pellets) and water were provided *ad libitum*. Healthy and sexually matured animals were utilized for the experiments. The experiments conducted had clearance from Institutional Animal Ethics Committee.

3.2 Test Chemicals

3.2.1 Laboratory reagents

MTZ, 1-[2-hydroxyethyl]-2-methyl-5-nitroimidazole; (CAS No.443-48-1) manufactured by J B Chemicals and Pharmaceutical Ltd, India. Mitomycin C (MMC) was obtained from Cadila Pharmaceutical, India. Giemsa’s stain and Eosin Y was obtained from HiMedia Laboratories. Artesunate (CAS NO: 83507-69) manufactured by Oscar remedies, India. Vitamin C and colchicine were purchased from Sigma Chemical (St Louis, MO). Normal melting point (NMP) agarose, low melting point (LMP) agarose, di-sodium salt of ethylene diamine tetra acetic acid (EDTA), Tris buffer, ethidium bromide (EtBr), Triton X-100, sodium hydroxide, sodium chloride, acetic acid and methanol were procured from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). DMSO [CAS No: 67-68-5] manufactured by Merck. Phosphate buffered saline (Ca2+, Mg2+ free; PBS); Mumbai, India. Trichloroacetic acid (Batch No: 451096), Titan Biotech Limited; 2-Thiobarbituric acid (CAS NO: 504-17-6), Hi-Media laboratories. Tris HCl, Pyrogallol, DTNB purchased from from Sisco Research Laboratories Pvt. Ltd (Mumbai, India), Curcumin (CAS NO:458-37-7) bought from Sigma chemicals.

The buffer for stain, reagent solutions and stains were always prepared in double-distilled water.

3.2.2 Drugs

Two common pharmaceuticals namely metronidazole, and artesunate were used in the present study. The chemical structure is given in Figure-1.
3.3 Methodology

Information on three levels of mutation, e.g., gene, chromosome, and cellular apparatus necessary for chromosome segregation, is necessary to provide broad coverage of the mutagenic and presumably carcinogenic potential of a chemical or radiation. In this regard, structural chromosome aberration assay, micronucleus assay and sperm shape abnormality assays are widely used to measure genotoxicity in somatic and/or germinal cells. The *in vivo* test is especially relevant to assessing genotoxicity hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes, and is also useful in further investigation of a mutagenic effect detected by an *in vitro* genotoxicity test (Krishna and Hayashi, 2000).

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Chemical</th>
<th>Chemical Name and Formula</th>
<th>Chemical Formula</th>
<th>CAS.No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Metronidazole</td>
<td>1-[2-hydroxyethyl]-2-methyl-5-nitroimidazole (C₆H₉N₃O₃)</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>443-48-1</td>
</tr>
<tr>
<td>2.</td>
<td>Artesunate</td>
<td>3R,5aS,6R,8aS,9R,10S,12R,12aR)-Decahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano(4,3-j)-1, 2- benzodioxepin-10-olhydrogen succinate (C₁₉H₂₈O₈)</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>83507-69</td>
</tr>
</tbody>
</table>

Figure 1:
Figure showing the chemical structure of metronidazole and artemesunate.
3.3. Methodology

Pharmaceutical agent selected for the study

1. Metronidazole
2. Artesunate

Drug-mutagen interaction studies

Gamma radiation (0.5 Gy)

X-ray (0.5 Gy)

Endpoints investigated

- Chromosomal aberration (CA), Micronucleus assay (MN), Sperm head abnormality assay (SHA), Comet assay
- Biochemical estimations (TBARS, GSH and SOD)

Antioxidants vitamin C (500 mg/kg) and curcumin (8 mg/kg bw) were administered prior to the treatment of test drug alone and in combination with gamma radiation and X-ray for 5 consecutive days.

Figure 2:
Schematic diagram showing test drugs and treatment.

3.3.1. Chromosome aberration assay

Under *in vivo* conditions, the genotoxicity assessment and in particular, the clastogenic potential of an agent is evaluated using the chromosome aberration assay (Preston, *et al*., 1987; Giri, *et al*., 2002a, 2002b; Cuenca and Ramirez, 2004; Boltina, 2007).

Very few agents e.g., ionizing radiation, bleomycin induce direct DNA breakage. These agents induce, at the time of exposure, chromosome-type chromosomal aberrations (involving the same locus on both sister chromatids on one or multiple chromosomes) in cells in the G0/G1 phase of the cell cycle and chromatid-type (involving only one sister chromatid).
chromatid of any one chromosome or more chromosomes) chromosome aberrations in cells in the S/G2 phase (Levy, et al., 2007). Operationally, these agents are classified as S-phase-independent clastogens.

The second mechanism is the one most likely to be applicable to many human population-monitoring studies as it represents the most common process by which clastogenic chemicals induce chromosome aberrations (Natarajan and Obe, 1980). Operationally, these agents are classified as S-phase-dependent clastogens. S-dependent agents are agents that induce DNA damage which require DNA synthesis before being expressed in the first metaphase post-exposure, or in the second metaphase as chromatid-type chromosome aberrations if the exposure took place in G2. Protocol:

Standard flame-drying technique was followed for metaphase preparations. Experimental animals were injected (i.p) with colchicine (4 mg/kg) 1.5 h prior to sacrifice. Bone marrow cells were collected from the femora by flushing in KCl (0.56% at 37°C for 18 min). The material was then centrifuged at 1000 rpm for 5 min, fixed in aceto-methanol (acetic acid: methanol, 1:3). Centrifugation and fixation (in cold) were repeated twice at an interval of 30 min. The material, re-suspended in a small volume of the fixative, was dropped on to chilled slides, flame-dried and stained in the following day in 5% giemsa for 10 min, air-dried and mounted in DPX mountant. One hundred good metaphase spreads were examined per animal. CA were classified into various categories like breakage of chromatid and isochromatid types, gaps of chromatid and isochromatid types, exchanges and sister chromatid unions (Figure -3).

Mitotic index were calculated by counting total dividing cells per thousand non-dividing cells. Mitotic index is the percent ratio of number of dividing cells to the total number of cells observed. The MI was significantly lower than that from the solvent control indicating a decrease in the number or rate of dividing cells, suggesting cytotoxicity.
Mitotic index (MI) = \[ \frac{\text{Dividing Cells}}{\text{Total number of cells studied}} \times 100 \]

Total aberrations were expressed as mean value ± S.E. In addition to total aberrations, percent aberrant cells were also evaluated out of the total 100 metaphases studied and suppression percentage was calculated as per the formula provided below:

\[
\text{Suppression Percent (\%)} = \frac{100 - \left( \frac{\text{Total aberrant cells studied induced by combination Treatment with antioxidant and test drugs}}{\text{Total aberrant cells induced by test drug alone}} \right) \times 100}{\text{(Total aberrant cells induced by test drug alone)} \times 100}
\]
Figure 3:
Photomicrographs of murine bone marrow metaphase spread showing different types of chromosomal aberration induced by test drugs alone and in various treatment combinations. A, C, D and G: Chromatid break; B and I: Isochromatid break; B: exchange; F and G: Sister chromatid union; E: Normal metaphase plate.
3.3. Methodology

3.3.2. Micronucleus assay

Evaluation of micronucleus frequency is the primary test in a battery of in vivo genotoxicity tests and recommended by the regulatory agencies around the globe to be conducted as part of product safety assessment (Krishna and Hayashi, 2000). To test somatic mutagens in vivo, bone marrow micronucleus assay has been used extensively (Giri, et al., 2002a, 2002b; Grujicic, et al., 2007; Donmez-Altuntas, et al., 2007).

Thus, an increased frequency of micronucleated cells is a biomarker of genotoxic effects that can reflect exposure to agents with clastogenic (chromosome breaking; DNA as target), or aneugenic (aneuploidogenic; effect on chromosome number; mostly non-DNA target) mode of action.

Protocol:

After the appropriate time of treatment, the animals were sacrificed by cervical dislocation. Bone marrow smears and staining were done following the method of Schmid (1976) with minor modifications (Giri, et al., 2002a, 2002b). Both the femora were removed and adhering tissues were cleared. Then the epiphyses were cut and the marrow was flushed into a centrifuge tube with 0.56% KCl (37° C) from a syringe. The marrow was gently agitated and immediately centrifuged at 1000 r.p.m. for 5 minutes. The supernatant was decanted off and the precipitate resuspended in a little volume of KCl. A drop of the suspension was taken on a clean grease free slide and allowed to stand for 30 – 40 seconds. Then a smear was made, air-dried, fixed in absolute methanol for 10 minutes and kept in dust free condition until staining. Staining was done in the following day in 5% buffered (pH 7.0) Giemsa. The slides were mounted with cover slip using DPX mountant. Scoring of the slides was done under the microscope using oil immersion. The polychromatic erythrocytes (PCEs) stain light blue to gray and normochromatic
erythrocytes (NCEs) stain light orange to straw yellow. A total of 3000 cells examined per animal. Micronucleated PCEs, NCEs and other nucleated cells (Figure-4) were used as indicator of genotoxicity in bone marrow cells.
Figure 4:
Photomicrograph of murine bone marrow cells i.e., polychromatic erythrocytes and normochromatic erythrocyte with micronucleus induced by test drug alone and in various treatment combination. A, I: Normal NCE; B, D, E, F and L: PCE with MN; G: Normal PCE; C, H, J, K: NCE with MN.
3.3.3 Sperm head abnormality assay

Sperm abnormality test is a sensitive and reliable endpoint to identify chemicals that induce spermatogenic dysfunction (Wyrobek and Bruce, 1978; Lock and Soares, 1980), and has been included in a battery of genotoxicity tests by many authors (Chauhan, et al., 2000; Rai and Vijayalaxmi, 2001, Giri et al., 2002a, 2002b). Although the obvious mechanism is not known, a correlation between germ cell mutagens and increased frequency of sperm shape alterations has been demonstrated by many authors (Vine, 1996; Saleh, et al., 2002, Zitzmann, et al., 2003; Hsu, et al., 2003). Wyrobek, et al. (1983) in a review estimated that virtually all murine germ cell mutagens tested so far induced sperm shape abnormality in mice.

Protocol:

Healthy male mice were sacrificed by cervical dislocation, 24hr and 35 days after the last dose of treatment, and dissected out. Both the cauda epididymus were removed and placed in a watch glass containing 1 ml of phosphate buffered saline (pH 7.2). The sperms were collected by disrupting the cauda epididymis and the suspension obtained was filtered through two layers of muslin cloth to remove tissue debris. Smears were made in clean grease free slides taking a drop of the material, air-dried, fixed in absolute methanol for 10 minutes, and stained in 0.1% aqueous eosin-Y solution in the following day. Slides were mounted in DPX before observation. At least one thousand sperms were analyzed per animal. Only the head morphology was examined for the present study. Abnormalities were categorized using the criteria as close to those described by Wyrobek and Bruce (1975) and modified (Bhunya and Pati, 1988; Giri, et al., 2002a, 2002b). Abnormal sperms had forms readily recognizable as amorphous, beak, hooked, triangular, banana-shaped, dwarf and giant (Figure–5).
3.3. Methodology

Total sperm Count was calculated by scoring cells in hemocytometer. Mutation factor and mutation indices were also calculated as given below.

\[
\text{Mutation factor (MF)} = \frac{\text{Frequency of abnormal sperm heads (Treated)}}{\text{Frequency of abnormal sperm head (Control)}}
\]

\[
\text{Mutation Index (MI)} = \frac{\text{Frequency of abnormal sperm heads (Treated- Control)}}{\text{Frequency of abnormal sperm heads (Control)}}
\]
Figure 5:
Photomicrograph of murine sperms showing abnormal head morphology induced by test drug alone and in various treatment combinations. A: showing normal head morphology; B, I, J, N: Amorphous; C: Hooked; D: Pin headed; E, F: Beaked; G, H, K, M: Banana; O: Hooked; P: Beaked; L: Hooked.
Biochemical assays: After sacrificing mice, liver was removed, weigh and cleaned. Tissue homogenate (10%) was prepared in ice cold normal saline and centrifuged for 10min at 3000 rpm. The supernatant is used for performing biochemical attributes. All biochemical assays were performed with a Thermo Scientific (Genesys-20) spectrophotometer.

3.3.4 Lipid peroxidation:

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process of free radicals induced damage to the lipids in cell membranes, resulting in cell damage. TBARS are expressed in terms of malonaldehyde (MDA) equivalents. (Kwon and Watts, 1964). Assays of TBARS measures MDA in the sample. It was also reported that lipid peroxidation products such as malondialdehyde forms adduct with cellular DNA (Mittal, et al., 2001). Malondialdehyde, formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of the peroxidation reaction. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a light pink color species absorbing at 535 nm. The presence of double bond adjacent to a methylene group makes the methylene C–H bonds of polyunsaturated fatty acid (PUFA) weaker and therefore the hydrogen becomes more prone to abstraction. While lipid peroxidation is not initiated by \( O_2^- \) and \( H_2O_2 \), \( \cdot OH \), alkoxy radicals (RO\(^-\)), and peroxy radicals (ROO\(^-\)) result in initiating the lipid peroxidation. This can lead to a self perpetuating process since peroxy radicals are both reaction initiators as well as the products of lipid peroxidation. Lipid peroxy radicals react with other lipids, proteins, and nucleic acids; thereby the transfer of electrons and bringing about the oxidation of substrates. Cell membranes, which are structurally made up of large amounts of PUFA, are highly susceptible to oxidative attack and, consequently, changes in membrane fluidity, permeability, and cellular metabolic functions result.
Biological specimens contain a mixture of thiobarbituric acid reactive substances (TBARS), including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress.

Protocol: Lipid peroxidation was assayed by the method of Rehman, 1984 and expressed as nmol malonaldehyde/g wet tissue. In brief, 1 ml of homogenate was incubated at 37° ±0.5 °C for 2 hr. To each sample, 1 ml of 10% w/v trichloroacetic acid was added. After thorough mixing, the mixture was centrifuged at 2000 rpm for 10 min. To 1 ml of supernatant, an equal volume of 0.67% thiobarbituric acid was added and kept in boiling water bath for 10 min. After cooling, it was diluted with 1 ml of distilled water (DW). The absorbance was read at 535 nm.

3.3.5 Reduced glutathione:

Glutathione is a tripeptide found in almost all mammalian cells. One of the residues of the tripeptide, cysteine, contains a sulfhydryl (-SH) group. The -SH group is key to nearly all reactions of glutathione (GSH). GSH provides reducing equivalents to the cell under conditions of oxidative stress and in the process, is oxidized to glutathione disulfide (GSSG). In this way, glutathione maintains a reducing intracellular environment and protects cells from oxidative damage. Thus, GSH is regarded as a small antioxidant molecule. GSH is a strong reducing agent owing to the presence of -SH group. It can donate electrons to species and in the process becomes oxidized (redox reaction). The antioxidant properties of GSH are due to this electron-donating nature of GSH.

Under conditions of oxidative stress, GSH is consumed in scavenging free radicals and removing ROS. GSH is a critical determinant of tissue susceptibility to oxidative damage and the depletion of hepatic
GSH has been shown to be associated with an enhanced toxicity to chemicals (Ko, et al., 1995).

**Protocol:** A known weight of tissue ranging from was homogenised in 5 to 8 ml of 0.02 M EDTA and then 4 ml of cold distilled water was added to it. After mixing it well, 1 ml of 50 % TCA was added and shaken intermittently for 10 min using vortex mixer. After 10 minutes the content were transferred to centrifuge tubes (rinsed in EDTA) and centrifuged at 6000 rpm for 15 minutes. Following centrifugation, 2 ml of the supernatant was mixed with 4 ml of 0.4 M tris buffer (pH 8.9). The whole solution was mixed well and 0.1 ml of 0.01 M DTNB was added to it. The absorbance was read within 5 minutes of addition of DTNB at 410 nm against a reagent blank with no homogenate.

**Calculation:**

GSH (tissue) was calculated from the following expression and expressed as μmol/g wt of tissue.

\[
\text{GSH (Co)} = \frac{A}{E} \times D
\]

Where,

- \(E\) = Extinction coefficient of DTNB blood
- \(A\) = Absorbance at 410 nm
- \(D\) = Dilution factor
- \(Co\) = Concentration of Glutathione

**3.3.6 Superoxide dismutase:**

The superoxide dismutases (SODs) are the first and most important line of antioxidant enzyme defense systems against ROS and particularly superoxide anion radicals. SOD is an important enzyme family in living cells for maintaining normal physiological conditions and for coping with
3.3. Methodology

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stress. The action of SOD therefore is to protect the biological integrity of the cells and tissues against harmful effects of superoxide free radical.

Protocol:

The supernatant was assayed for superoxide dismutase activity by following the inhibition of pyrogallol auto oxidation. 100 μl of cytosolic supernatant was added to tris HCl buffer, pH 8.5. The final volume of 3 ml was adjusted with the same buffer. At last 25 μl of pyrogallol was added and changes in absorbance at 420 nm were recorded at 1 minute interval for 3 minutes. The increase in the absorbance at 420 nm after the addition of pyrogallol was inhibited by the presence of SOD.

One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol auto oxidation per 3 ml of assay mixture and is given by the formula:

Unit of SOD per ml of sample = \[ 100 \times \frac{(A-B)}{(Ax50)} \]

Where,

\[ A = \text{Change in absorbance per minute in control.} \]
\[ B = \text{Change in absorbance per minute in test sample.} \]

3.3.7 Single cell gel electrophoresis:

The alkaline single-cell gel electrophoresis (SCG) assay, a novel procedure for evaluating DNA lesions (single strand breaks and alkali-labile sites), involves application of an electrical current to cells, which results in the transport of DNA fragments out of the nucleus. The image of DNA migration obtained resembles a comet with a head and a tail, hence the term comet assay (Klaude, et al., 1996; Singh and Stephens, 1996). Since the DNA damage induced by toxic agents is often tissue- and cell-specific, SCG is very useful because it can detect DNA lesions in individual cells obtained under a variety of experimental conditions; the technique can also be used to evaluate DNA repair (Tice, 1995; Petras, et al., 1995). A
significant advantage of the SCG assay is its applicability to any eukaryotic organism and cell type.

Figure 6:
Schematic diagram showing comet assay protocol. (Courtesy: IITR, Lucknow)

Protocol
The DNA damage studies were carried out following the comet assay according to the method of (Singh, et al., 1988) with modifications (Tice, et al., 2000). Mice femurs were dissected out and bone marrow was aspirated from each femur into PBS solution. The cell suspension (25 μl)
was mixed 1:10 with 250 μl molten low melting point (LMP) agarose, and samples of 85 μl of the mixture were rapidly spread on Comet Slides. Slides were prepared in triplicates per concentration. Slides were immersed in cold lysis solution at pH 10. The lysis solution consisted of 2.5M NaCl, 100mM Na₂EDTA, 10mM Trizma base, 1% TritonX100, 10% DMSO and kept at 4°C for 60min. After lysis the DNA was allowed to unwind in the electrophoresis buffer (300mM NaOH: 1mM Na₂EDTA at pH 13.5) for 20 min. This was followed by electrophoresis conducted at a constant voltage of 24V and 300mA at 4°C. Slides were neutralized in 0.4M Tris buffer (pH 7.5) for 5 min and finally rinsed in distilled water. Each experiment was repeated twice. The slides were stained with EtBr (20μgm per L) and rinsed in distilled water to wash off excess stain. Slides were scored using image analysis system (Kinetic imaging; Andor Technology, Nottingham, UK) attached to a fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate filters. The microscope was connected to a computer through a charge coupled device (CCD) camera to transport images to software (Komet 5.5) for analysis. The final magnification was 100X. Among the comet parameters we report the percent of DNA in the tail (tail DNA). This will give a clear indication of the extent of DNA damage induced by the test chemical. Images of 150 (50x3) cells per concentration were analyzed.
Figure 7:
Photomicrograph of comet tails induced by test drug alone and in various treatment combinations in mice bone marrow cells. A,B: Normal cell; C,D,E,F,G,H,I,J,L: Cells with comet tail; K: Apoptotic cell.
3.3.8 Statistical analysis:

All the data were represented as mean±S.E. The corresponding graph of the tables were represented by histograms and line diagram. In the present work the variables considered were the test drugs alone and in various treatment combination and the other variables included the genetic changes (chromosomal aberration, micronucleus formation, sperm head abnormality and DNA damage), biochemical changes (lipid peroxidation, reduced glutathione and superoxide dismutase).

ANOVA was used to determine the significance of parameters. Since there is no significant heterogeneity of variance, one way analysis of variance was used to test whether there was evidence of any difference between mean of groups. Pair wise comparison of significance between the different groups was determined by Turkey’s test. ANOVA values were calculated using GraphPad Prism, version 4.03 (GraphPad Inc., San Diego, CA, USA). A p-value of <0.05 was considered to be significant.