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Abstract
Epirubicin, a semi-synthetic anthracycline antibiotic, is widely prescribed singly or in combination therapy for the treatment of various types of cancers. However, the reported occurrence of second malignancy in epirubicin pre-treated cancer survivors has necessitated its cytogenotoxicity testing. Therefore, the clastogenic potential of epirubicin was assessed here from bone marrow cells of Swiss mice after single intraperitoneal administration of the drug. Each of the three tested doses of epirubicin (2, 4 and 6 mg kg⁻¹ b. w.) induced significantly high percentages (p ≤ 0.01) of aberrant metaphases and chromosomal aberrations (excluding gaps) at 24 h post-treatment and significant increase (p ≤ 0.05) in the frequency of micronucleus in polychromatic erythrocytes at 30 h post-treatment. Thus, epirubicin was highly clastogenic to bone marrow cells of Swiss mice. Its interference in the activity of topoisomerase-II and its free radical generation potential were attributed to its clastogenicity. Such clastogenic effects of epirubicin might have been the cause of recurrence of second malignancy in post-chemotherapeutic cancer survivors.

Keywords: Epirubicin; Anthracycline antibiotic; Chromosome aberration; Micronucleus; Bone marrow cells; Swiss mice.

Introduction
Anthracycline-antibiotics are widely prescribed as anti-neoplastic agents for the successful treatment of various types of cancers, particularly leukaemia (Bhutani et al., 2002), solid tumours (Minotti et al., 2004; Wasowska et al., 2005) and breast cancer (Gianni et al., 2009). Epirubicin (EPI), a semi-synthetic anthracycline antibiotic, isolated from Streptomyces peucetius var. caesius, is the 4’-epimer of doxorubicin (DOX). The sugar moiety of EPI differs from the natural daunosamine sugar in steric configuration of the hydroxyl group bearing C-4 in inverted position, thereby forming the L-arabino configuration of EPI instead of the L-lyxo in DOX. The anthracycline ring of EPI is lipophilic and its saturated end of the ring system contains the hydroxyl group adjacent to the amino sugar by producing a hydrophilic centre. Both the acidic function in phenolic ring and basic function in amino group confer EPI as amphoteric (Plosker and Faulds, 1993; Minotti et al., 2004; Wasowska et al., 2005). In cancer chemotherapy, EPI is prescribed singly (Bastholt et al., 1996; Lopez et al., 2002; Barni et al., 2004) or as a major component in combination chemotherapy regimen (de Azambuja et al., 2009) for the effective treatment of various types of cancers. Moreover, dose-response relationship of EPI (Focan et al., 1993; Bastholt et al., 1996) with provision of its dose intensification (Lanchbury and Habboubi, 1993) has facilitated the treatment of different malignancies (The French Epirubicin Study Group, 2000; Lopez et al., 2002).

However, treatments with EPI not only exhibit multiple side effects (Minotti et al., 2004) but also induce an increased risk of secondary leukaemia (Campone et al., 2005; Praga et al., 2005; Mays et al., 2010). These causes of concern necessitated the assessment of the cytogenotoxic potential of EPI. But, there is sporadic occurrence of such test reports (Othmann, 2000; Brumfield and Mackay, 2002; Lehman et al., 2003, Buschini et al., 2003; Sen et al., 2010), which have been carried out either in vitro, or in lower organisms in vivo, or after chronic exposures of EPI along with other drugs. The results of such studies are fragmentary, inadequate and inconclusive. Therefore, the present study has been undertaken to evaluate the clastogenic potential of the lone drug EPI from bone marrow cells of Swiss mice after a single intraperitoneal exposure of three different doses.

Materials and Methods
Test animal
Swiss albino mice (Mus musculus), initially procured from M/S Ghosh Enterprise, Kolkata, India, were reared in the animal house of the
department in hygienic condition. The animal house was maintained at an optimal temperature of 23±2°C with a 12 h light/dark cycle. The mice were provided with balanced diet and drinking water ad libitum. Healthy mice of approximately 20 g b. w. each were selected for experiments from among the reared stock of mice.

Test chemicals
Alrubicin® (Epirubicin hydrochloride injection), manufactured by Alkem Laboratories Ltd., Mumbai, India, was used as the test chemical. It is a semi-synthetic anthracycline cytotoxic antibiotic. Lodoxan (Cyclophosphamide injection), manufactured by Dabur Pharma, New Delhi, India, was used as the positive control chemical. Sodium Chloride (NaCl) was used as the negative control.

Dose of the chemicals and route of administration
EPI is generally prescribed in combination with other drugs, such as cyclophosphamide + epirubicin + 5-fluorouracil (CEF). The FDA approved doses are: (1) CEF 120 (with EPI @ 60 mg/m²) i. v. on days 1, 8 repeated every 28 days for six cycles, or (2) CEF 100 (with EPI @ 100 mg/m²) on day 1 and repeated 21 days for six cycles. Therefore, in accordance with human therapeutic dose, three different doses of EPI (2, 4 and 6 mg/kg b. w.) were selected for testing on mice by taking the relative b. w. and surface area of mouse to that of an adult person into considerations. Cyclophosphamide (CY) 40 mg/kg b. w. of mice was selected as the dose of positive control chemical and 0.9% sodium chloride @ 10 ml/kg b. w. was used as the negative control. Dilution of the chemicals was made in such a way that the volume of each treatment was maintained to 1 ml/100 g b. w. of mice. The tests were undertaken after single intraperitoneal treatment of the chemical to mice for one cell cycle duration which is suitable for the assessment of clastogenicity.

Endpoints studied
1. Mitotic metaphase chromosome aberration (CA) study from bone marrow cells at 24 h post-treatment.
2. Micronucleus Test (MNT) from polychromatic erythrocytes (PCEs) at 30 h post-treatment.

Experimental protocol
Sixty healthy mice (30 females and 30 males), approximately 20 g b. w. each, were selected and randomly divided into five groups of 12 mice (6 females and 6 males) each. One group, the negative control group, was treated with 0.9% sodium chloride @ 1 ml/100 g b. w. Another group, the positive control group, was treated with CY@ 40 mg/kg b. w. Rest three groups were treated with EPI @ 2, 4 or 6 mg/kg b. w. At 24 h post-treatment, six mice (3 females and 3 males) from each of the treated groups were employed in CA study and the rest six mice of each group (3 females and 3 males) were utilized in MNT at 30 h post-treatment, which is required for enucleation of the main nucleus and formation of PCE.

Procedure
Slides for mitotic metaphase CA study from bone marrow cells at 24 h post-treatment were prepared following the colchicine- sodium citrate hypotonic- methanol, glacial acetic acid- flame drying- Giemsa technique of (Choudhury et al., 2000). More than 150 well spread metaphases were scanned from each animal under binocular research microscope. Aberrant metaphases and different CAs in them were recorded. Percentages of aberrant metaphases (including metaphases with only gaps) and aberrations (excluding gaps) per 100 metaphases were calculated. Each pulverized metaphase was equated to 10 aberrations in calculation.

Slides for MNT were prepared from bone marrow cells at 30 h post-treatment following the simple procedure of (Choudhury et al., 2000). About 2000 blue tinged PCEs were scanned from each animal under binocular research microscope and the MN in them were recorded. MN per 1000 PCEs was calculated.

Statistical analysis
Averages with standard deviations were calculated from the recorded data at the two different endpoints of different groups of mice, and were pooled together for the male and female mice of each group separately. The calculated average data of different endpoints for the positive control group and that of the EPI-treated groups were compared with the respective data of the negative control groups of mice. Statistical significance of the differences among the respective pooled data at different levels (p ≤ 0.05 or p ≤ 0.01) were ascertained from the tables of (Kastenbaum and Bowman, 1970) which were prepared specifically for mutation studies.
Results
The percentages of aberrant metaphases in the female and male mice of the negative control group were 2.57±0.66 and 3.01±0.47, and that of the positive control group were 63.33±0.35 and 51.29±0.68 respectively. The induced percentages of aberrant metaphases by the three different doses of EPI (2, 4 or 6 mg/kg b. w.) in the female and male mice were 12.44±2.14 and 22.22±8.49, 19.99±2.90 and 24.66±4.66, and 25.33±6.96 and 29.77±5.09, respectively. The increased percentages of aberrant metaphases in the positive control group and in the EPI-treated groups of mice were significantly higher (p ≤ 0.05 or p ≤ 0.01) than that of the respective negative control group of mice (Table 1).

Average CAs (excluding gaps) per 100 metaphases in female and male mice of the negative control group were 0.64±0.03 and 0.33±0.29, and that of the positive control group were 110.65±4.35 and 81.31±3.35 respectively. EPI 2, 4 and 6 mg/kg-induced CAs (excluding gaps) per hundred metaphases in female and male groups of mice were 33.99±8.96 and 29.55±7.89, 40.88±3.67 and 55.99±1.15, and 72.88±10.66 and 70.22±11.49, respectively. The positive control chemical and all the three different doses of EPI-induced CAs were increased significantly (p ≤ 0.01) from that of the respective negative control mice (Table 1).

Table 1: Cyclophosphamide (CY)- and Epirubicin (EPI)-induced chromosomal aberrations (CAs) in bone marrow cells of mice at 24 h post-treatment.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Dose (mg Kg⁻¹ body weight)</th>
<th>Number of mice treated &amp; gender</th>
<th>Number of metaphases examined</th>
<th>Number of aberrant metaphases</th>
<th>Type and number of CAs</th>
<th>Total CAs (excluding gaps)</th>
<th>Average percentage of aberrant metaphases ±SD</th>
<th>Average CAs (excluding gaps) per 100 metaphases ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl (Negative control)</td>
<td>10 ml kg⁻¹</td>
<td>3f</td>
<td>465</td>
<td>12</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3m</td>
<td>596</td>
<td>18</td>
<td>15</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>EPI</td>
<td>2</td>
<td>3f</td>
<td>450</td>
<td>56</td>
<td>12</td>
<td>20</td>
<td>3</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3m</td>
<td>450</td>
<td>110</td>
<td>11</td>
<td>93</td>
<td>4</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3f</td>
<td>450</td>
<td>90</td>
<td>19</td>
<td>45</td>
<td>2</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3m</td>
<td>450</td>
<td>111</td>
<td>13</td>
<td>128</td>
<td>1</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3f</td>
<td>500</td>
<td>129</td>
<td>24</td>
<td>115</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3m</td>
<td>450</td>
<td>134</td>
<td>13</td>
<td>145</td>
<td>1</td>
<td>--</td>
</tr>
<tr>
<td>CY(Positive control)</td>
<td>40</td>
<td>3f</td>
<td>377</td>
<td>239</td>
<td>56</td>
<td>75</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3m</td>
<td>427</td>
<td>219</td>
<td>87</td>
<td>104</td>
<td>22</td>
<td>60</td>
</tr>
</tbody>
</table>

SD - standard deviation, Frag - fragment, Min - minute, Pul - pulverization, Brk - break, f - female, m - male, (*) p < 0.05, (**) p < 0.01

Average MN per thousand PCEs in the female and male mice of the negative control and positive control groups were 1.99±0.77 and 3.66±0.83, and 17.15±1.05 and 29.85±4.02, respectively. Increase in the induction of MN in the positive control group of mice was statistically highly significant (p ≤ 0.01). EPI 2, 4 and 6 mg/kg-induced average MN per 1000 PCEs in the female and male mice were 9.06±0.61 and 8.35±0.84, 9.35±1.63 and 8.57±2.04, and 9.87±0.65 and 9.85±1.15, respectively. All the three doses of EPI-induced MN were increased from that of the respective negative control group of mice. Such increases in MN frequency in EPI 2 and 4 mg/Kg treated female mice are statistically significant (p ≤ 0.05), when compared to that of the female mice of the negative control group. Although such increases in the EPI-induced MN in rest groups of mice are statistically not significant, they are almost on the borderline (Table 2).
Table 2: Cyclophosphamide (CY)- and Epirubicin (EPI)-induced MN in PCEs of mice at 30h post-treatment.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Dose (mg kg(^{-1}) body weight)</th>
<th>Number of mice treated &amp; gender</th>
<th>Number of PCEs observed</th>
<th>Number of PCEs with MN</th>
<th>Total number of MN</th>
<th>Average MN per 1000 PCEs ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl</td>
<td>10 (ml kg(^{-1}))</td>
<td>3f</td>
<td>6228</td>
<td>12</td>
<td>12</td>
<td>1.99±0.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3m</td>
<td>6365</td>
<td>22</td>
<td>23</td>
<td>3.66±0.83</td>
</tr>
<tr>
<td>EPI</td>
<td>2</td>
<td>3f</td>
<td>6292</td>
<td>55</td>
<td>57</td>
<td>9.06±0.61**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3m</td>
<td>6439</td>
<td>44</td>
<td>54</td>
<td>8.35±0.84</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3f</td>
<td>6337</td>
<td>55</td>
<td>59</td>
<td>9.35±1.63*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3m</td>
<td>7035</td>
<td>59</td>
<td>60</td>
<td>8.57±2.04</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3f</td>
<td>6994</td>
<td>52</td>
<td>69</td>
<td>9.87±0.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3m</td>
<td>6495</td>
<td>51</td>
<td>64</td>
<td>9.85±1.15</td>
</tr>
<tr>
<td>CY (Positive control)</td>
<td>40</td>
<td>3f</td>
<td>10841</td>
<td>171</td>
<td>193</td>
<td>17.15±1.05**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3m</td>
<td>6066</td>
<td>155</td>
<td>177</td>
<td>29.85±4.02**</td>
</tr>
</tbody>
</table>

SD - standard deviation, PCEs - polychromatic erythrocytes, MN - micronucleus, f - female, m - male, 
(*) p ≤ 0.05, (**) p ≤ 0.01

**Discussion**

CY, a covalent DNA-binding agent, has reportedly induced CAs, MN, sister chromatid exchanges, heritable translocations, specific locus mutations and dominant lethal mutations in both mammalian and non-mammalian test systems, which have been updated for a review (Anderson et al., 1995). Moreover, its use as positive control in cytogenotoxicity tests has been recommended (Krisna et al., 1995). Induction of significantly (p ≤ 0.01) high percentages of aberrant metaphases, CAs (excluding gaps) per hundred metaphases and MN per thousand PCEs by CY 40 mg/kg b. w. of mice, in the present study (Table 1 and 2), are in complete agreement with its earlier reported clastogenicity in mouse bone marrow.

Induction of significantly high percentages of aberrant metaphases (p ≤ 0.05 or p ≤ 0.01) and CAs (excluding gaps) per 100 metaphases (p ≤ 0.01) by all the three tested doses of EPI (2, 4 and 6 mg/kg) in bone marrow cells of Swiss mice, when compared to that of the negative control mice, indicated that EPI was highly clastogenic to mouse bone marrow cells. EPI-induced structural chromosomal aberrations were mostly chromatid breaks, fragments, minutes and a few pulverized metaphases (Table 1). Both, the percentages of aberrant metaphases and CAs were found increased along with the increase in the doses of EPI. This dose-dependent increase in the clastogenicity of EPI, in the present study, is in complete agreement with that of Othman (2000), reported on Chinese hamster cell line in vitro. EPI-induced clastogenicity was found higher in male groups of mice than the respective female mice in all the three doses, except in the induction of CAs by EPI 6 mg/kg (Table 1). EPI-induced MN in all the groups of mice treated with the different doses of EPI, were remarkably increased from that of the negative control mice (Table 2). Such increases are statistically significant (p ≤ 0.05) in the female mice that received EPI 2 and 4 mg/kg. However, in the rest groups of EPI-treated female and male mice, such increases are in the borderline. As micronuclei are generated out of the aberrated chromosomes, this increased induction of MN in all the EPI treated groups of mice strongly supported the contention that EPI was highly clastogenic.

EPI was reportedly mutagenic to Salmonella typhimurium, causing frame-shift and transition mutations (Brumfield and Mackay, 2002), and cytotoxic to Saccharomyces cerevisiae (Buschini et al., 2003). It caused somatic mutations and homologous recombinations in Drosophila melanogaster (Lehman et al., 2003), damaged DNA in CBA mice (Garaj-Vrhovac et al., 2003) and in the peripheral blood lymphocytes of patients under...
therapy (Barni et al., 2004; Sánchez-Suárez et al., 2008), and cytotoxic to breast cancer cell lines (Azab et al., 2005). However, there is a striking increase in the use of EPI, singly or in combination chemotherapy, for the successful treatment of sarcomas, particularly breast cancers. On the other hand, there is the risk of secondary leukaemia in cancer survivors pre-treated with EPI (Campone et al., 2005; Praga et al., 2005; Mays et al., 2010).

Although the prime objective of cancer chemotherapy is to kill the cancerous cells, their selective killing without affecting the non-cancerous cells is not feasible, as cancer chemotherapeutics are mostly not target specific, and moreover, they are cytogenotoxic (Choudhury et al., 2000; Pal et al., 2009). These drugs have been designed to affect and interfere in the genetic system of the cancerous cells, thereby killing the cells through various mechanisms. As EPI is clastogenic, during its systemic treatment to cancer patients, the non-cancerous cells of the patients exposed to the drug are also equally affected along with the cancerous cells. Non-cancerous cells with such gross effects on their genome might be eliminated and some of them with tolerable effects might be sustained. Since chromosomal aberrations are related to incidence of cancer (Rossner et al., 2005; Boffetta et al., 2007; Bonassi et al., 2008; Mays et al., 2010), induction of secondary leukaemia and other secondary cancers in post-EPI treated cancer survivors is, most plausibly, due to the accumulation of clastogenic effects of EPI on non-cancerous cells of cancer patients during chemotherapy. Therefore, assessment of the detail cytogenotoxic potential of EPI is essential to make it target specific and/or to modulate its effects (Azab et al., 2005).

Cytogenotoxicity of anthracycline antibiotics has been attributed to the inhibition of topoisomerase II (topo-II) activity and to the production of free radicals. These drugs disrupt the topo-II mediated DNA cleavage and religation reactions resulting in the formation of stabilized cleavage complexes leading to DNA strand breaks, major deletions and illegitimate recombinational events during DNA replication (Gewirtz, 1999). These drugs also reportedly induce intracellular production of free radicals, like superoxides, hydrogen peroxides and hydroxyl radicals (Doroshow, 1983), leading to oxidative DNA damages and other lesions on DNA (Anderson and Berger, 1994; Olinski et al., 1997). Therefore, use of antioxidant(s) along with EPI treatment might be useful in modulating its cytogenotoxicity.

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