Research Paper

Antineoplastic Activity of Chloroacetohydroxamic Acid in Combination with Bleomycin Against Ehrlich Ascites Carcinoma (EAC) in Mice

J. A. Khanam and A. Y. K. M. Masud Rana

The research work has been undertaken in order to investigate the antineoplastic activity of chloroacetohydroxamic acid (CHA) in combination with bleomycin against Ehrlich ascites carcinoma (EAC) in Swiss Albino mice. Results showed that combination of treatment inhibits the tumor growth to more than 90% as against 56 and 65% for CHA and bleomycin, respectively. The combination treatment increases the life span of EAC bearing mice to 70% as against 30 and 50% for CHA and bleomycin, respectively. The combination treatment also recovers the hematological parameters and alkaline phosphatase (ALP) activity of tumor bearing mice more precisely than do CHA and bleomycin, individually.

Key words: EAC, CHA, bleomycin
Khanam and Rana: Activity of chloroacetohydroxamic acid in combination with bleomycin

Introduction
Combination treatment is a new phase of cancer treatment, which expands the life span of cancer patient without additive toxicities (Wasserman et al., 1977). In designing a combination regimen, agents are chosen, which are individually active against the disease in full doses and have different mechanism of action (Raphael and George, 1984). Among the individual drugs, bleomycin is very familiar with unique pharmacological characteristics and its therapeutic use against a wide range of human malignancies (Barranco and Humphrey, 1971; Wheatley et al., 1974). However like other anticancer drugs, bleomycin is also toxic in nature. The usual dose limiting toxicity of bleomycin is pulmonary fibrosis and hyperpyrexia (Keim and Hong, 1981; Agre, 1974). However, bleomycin, because of its lack of myelosuppression, has proved to be attractive for combination treatment (Greenberg et al., 1982). Various attempts have been made so far to minimize the toxicity of bleomycin as well as to get better antitumorogenic effect by combination chemotherapy (Goliz and Kline, 1978). It is now known that activity of bleomycin is potentiated by some drugs such as enactrin and neocanactin containing hydroxamic acid structure (Inouye, 1988). Compounds containing hydroxamic acid functional group have been found to possess antitumor activity (Efird et al., 1979). Such types of studies with chloroacetohydroxamic acid (CHA) have been reported recently (Sur et al., 1997). Better results are therefore expected with the combined chemotherapy of bleomycin and CHA.

This paper comprises the investigation of antitumor properties of CHA in combination with bleomycin. Survival time and cell growth inhibition as well as the host toxicity of the blood parameters and alkaline phosphatase (ALP) activities of serum of tumor bearing mice have also been evaluated.

Materials and Methods
Synthesis of chloroacetohydroxamic acid (CHA): CHA was synthesized by the reaction of ethylchloroacetate (CH₂-CO-CH₂-Cl) and hydroxylamine (NH₂-OH) according to the procedure of Jones and Werner (1917). The compound was confirmed from elemental analysis data and melting point (107°C). Bleomycin sulphate was purchased from Lipincot, Japan and others from Sigma Chemicals (USA) and BDH Ltd. (Dugennam, U. K.).

Animals: Male Swiss A mice 6-8 weeks of age weighing 20-25gm were used (obtained from International DIARRhoea Disease Research Bangladesh). Animals were fed standard mouse pellet. Hind, Lever, Mumbai, India and water was given ad libitum.

Tumor cells: Ehrlich ascites carcinoma (EAC) cells were obtained through the courtesy of Chittaranjan National Cancer Research Center. Calcutta-2 and maintained by weekly intraperitoneal (i.p.) inoculation of 10⁶ cells mouse⁻¹ in the laboratory.

Drug treatment: A specified dose (25mg kg⁻¹) of CHA and bleomycin (0.3mg kg⁻¹) were administered by intraperitoneal (i.p.) injection. The drug was administered 24hr after inoculation of the mouse with 10⁵ EAC cells, bleomycin was injected after 3 hours of CHA injection. The control group was treated with 0.9% normal saline.

Cell growth inhibition: In vivo tumor cell growth inhibition (Sur and Ganguli, 1994) were carried out with CHA, bleomycin and in combination with CHA and bleomycin. Animals (in 4 group) were inoculated (i.p.) with test compounds started 24 hr, after inoculation and continued for 4 consecutive days. Animals were sacrificed on day 6 after transplantation and tumor cells were collected by repeated intraperitoneal wash with 0.9% saline. Viable tumor cell counts were made with a hemocytometer. Total number of viable cells per animal of the treated group was compared with those of control group.

Survival time: Survival time of EAC bearing mice treated with CHA (25mg kg⁻¹, i.p.), bleomycin (0.3mg kg⁻¹) and in combination with CHA and bleomycin were carried out for 10 days under similar experimental conditions as stated for previous experiment. Tumor weight was measured on each day from the day of EAC cell inoculation into mice. Mean survival time (MST), for each group containing 8 mice was noted. Survival time of treated group was compared with those of control group using the following calculation (Sur et al., 1984):

\[
\text{Percent increase of life span} = \left(\frac{\text{MST of treated group} - \text{MST of control group}}{\text{MST of control group}}\right) \times 100 \text{ - } 100
\]

Hematological studies: In order to detect the influence of CHA, bleomycin and CHA in combination with bleomycin on hematological status of EAC bearing mice, comparison was made among the five groups (n = 4) of mice on the 12th day, after tumor transplantation as cited in the literature (Khanam et al., 1987). The five groups comprised

1. EAC bearing mice treated with 0.9% normal saline only;
2. EAC bearing mice treated with CHA;
3. EAC bearing mice treated with bleomycin;
4. EAC bearing mice treated with CHA and bleomycin and
5. Normal mice served as control.

Treatment was continued for 10 consecutive days.

Blood was collected from individual mice for the experiment. Total counts of white blood cells (WBCs) and red blood cells (RBCs) as well as the hemoglobin (Hb) content were determined by standard methods (Rusia and Swaran, 1988) using cell diluting fluids and a hemocytometer. The differential count was carried out with Wright stain.

Alkaline phosphatase (ALP) activity: ALP activity in the serum of EAC cell bearing Swiss Albino mice of all such groups (treated with CHA, bleomycin and CHA in combination with bleomycin and untreated EAC bearing mice (n = 4)) were analyzed on day 12 of cell transplantation. The ALP activity was measured spectrophotometrically at 410nm according to the method reported by Telfer. 1993 using para-nitrophenyl phosphate (PNPP) as substrate, in glycine-sodium hydroxide buffer (pH = 10). Significance of the experiments were statistically evaluated (Ghosh, 1984) by students t-test and the level of significance is 0.05.

Results
Cell growth inhibition: The effects of CHA, bleomycin and CHA in combination with bleomycin on EAC cell growth on day 5 after tumour transplantation are shown in Table 1. Combination treatment for 4 days resulted 91% tumor growth inhibition where as individual treatment with bleomycin and CHA, inhibited the tumor growth only 85 and 68% respectively.

Survival time: Treatment with CHA, bleomycin and in combination, bearing mice resulted decrease in tumor weight as shown in Fig. 1. CHA potentiated the antitumor activity of
Table 1: Effect of Chloroacetohydroxamic acid (CHA), bleomycin and CHA in combination with bleomycin on inhibition of tumor cell growth (in vivo) in mice and survival of tumor bearing mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of cells mice⁻¹ (×10⁶) on day 6</th>
<th>% of cell growth inhibition</th>
<th>Mean survival time (MST) days</th>
<th>Percent increase of life span (PILS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.8% saline)</td>
<td>1.87 ± 0.12</td>
<td>20 ± 3</td>
<td>30 ± 3</td>
<td>90</td>
</tr>
<tr>
<td>CHA (25mg kg⁻¹·pl)</td>
<td>0.81 ± 0.19**</td>
<td>56.68</td>
<td>28 ± 2</td>
<td>90</td>
</tr>
<tr>
<td>Bleomycin (10.3mg kg⁻¹·pl)</td>
<td>0.64 ± 0.05**</td>
<td>68.77</td>
<td>30 ± 3</td>
<td>90</td>
</tr>
<tr>
<td>CHA (25mg kg⁻¹·pl) +</td>
<td>0.16 ± 0.04**</td>
<td>91.97</td>
<td>34 ± 4</td>
<td>70</td>
</tr>
<tr>
<td>Bleomycin (10.3mg kg⁻¹·pl)</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Results shown are mean values ± SEM. No. of mice per group are 6. **P< 0.001 when compared with control.

Table 2: Effect of CHA, bleomycin and CHA in combination with bleomycin on hematological parameters in tumor bearing mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Hb (gmld⁻¹·pl)</th>
<th>RBCx10¹²/ml</th>
<th>MCVx10¹² ml⁻¹·pl</th>
<th>Lymphocytes (%)</th>
<th>Neutrophils (%)</th>
<th>Monocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mice</td>
<td>14.60 ± 0.40</td>
<td>8.30 ± 0.18</td>
<td>9.0 ± 0.27</td>
<td>70 ± 2.40</td>
<td>28 ± 2.70</td>
<td>2 ± 0.64</td>
</tr>
<tr>
<td>(0.8% saline control)</td>
<td></td>
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</tr>
<tr>
<td>EAC bearing mice</td>
<td>8.56 ± 0.58**</td>
<td>4.01 ± 0.34**</td>
<td>28 ± 3.2**</td>
<td>41 ± 1.3**</td>
<td>57 ± 2.45**</td>
<td>3 ± 0.50</td>
</tr>
<tr>
<td>CHA (25mg kg⁻¹·pl)</td>
<td>10.6 ± 0.10**</td>
<td>5.9 ± 0.30**</td>
<td>15 ± 0.4**</td>
<td>42 ± 2.20**</td>
<td>56 ± 2.40**</td>
<td>2 ± 0.50</td>
</tr>
<tr>
<td>Bleomycin (0.3 mg kg⁻¹·pl)</td>
<td>11.0 ± 0.20**</td>
<td>5.8 ± 0.20**</td>
<td>8.28 ± 0.8</td>
<td>68 ± 2.30</td>
<td>30 ± 1.40</td>
<td>2 ± 0.50</td>
</tr>
<tr>
<td>CHA (25mg kg⁻¹·pl) +</td>
<td>12.0 ± 0.60</td>
<td>6.8 ± 0.60</td>
<td>9 ± 0.82</td>
<td>68 ± 1.63</td>
<td>30 ± 1.70</td>
<td>2 ± 0.50</td>
</tr>
<tr>
<td>Bleomycin (0.3 mg kg⁻¹·pl)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

No. of mice per group are 4. Results are mean ± SEM. **P< 0.001 when compared with control. EAC (Ehrlich ascites carcinoma) CHA (Chloroacetohydroxamic acid)

Table 3: Effect of CHA, bleomycin and CHA in combination with bleomycin on alkaline phosphatase (ALP) activity in the serum of EAC bearing mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>ALP activity x 10⁻³ amol PNPP hydrolyzed min⁻¹·m⁻¹ serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mice</td>
<td>12.87 ± 0.81</td>
</tr>
<tr>
<td>Control (EAC bearing mice)</td>
<td>5.01 ± 0.45**</td>
</tr>
<tr>
<td>CHA (25 mg kg⁻¹·pl)</td>
<td>7.72 ± 0.82**</td>
</tr>
<tr>
<td>Bleomycin (0.3 mg kg⁻¹·pl)</td>
<td>5.29 ± 0.45**</td>
</tr>
<tr>
<td>CHA (25 mg kg⁻¹·pl) +</td>
<td>10.49 ± 0.84</td>
</tr>
<tr>
<td>Bleomycin (0.3 mg kg⁻¹·pl)</td>
<td></td>
</tr>
</tbody>
</table>

No. of mice per group are 4. Results are mean ± SEM. **P< 0.001 when compared with control.

Fig. 1. Effect of CHA in combination with bleomycin treatment on tumor weight in mice Control □, CHA (25mg kg⁻¹·pl) ▲, Bleomycin (0.3 mg kg⁻¹·pl) ▲, CHA (25 mg kg⁻¹·pl) + Bleomycin (0.3 mg kg⁻¹·pl) ▲. Number of mice in each group was 6.

Fig. 2. Effect of CHA in combination with bleomycin treatment on survival time of Ehrlich ascites carcinoma (EAC) bearing mice. Control □, CHA (25mg kg⁻¹·pl) ▲, Bleomycin (0.3 mg kg⁻¹·pl) ▲, CHA (25 mg kg⁻¹·pl) + Bleomycin (0.3 mg kg⁻¹·pl) ▲. Number of mice in each group was 6.

Blood parameters: In tumor bearing mouse hematological parameters were found to be altered significantly from normal values except for monocyte on the 12th day of tumor transplantation. Bleomycin could recover all of these depleted parameters to normal except Hb and RBC values. These depleted Hb and RBC values were found to be recovered towards normal values after treatment with bleomycin in combination with CHA (Table 2).

ALP activity: Growth of EAC cells in mice showed a depletion of ALP activity in serum. Recovery of ALP activity of serum of tumor bearing mice was observed with CHA and bleomycin treatment. But significant recovery of enzyme activity was found in the serum of tumor bearing mice following CHA in combination with bleomycin.
combination with bleomycin treatment (Table 3).

Discussion
In the in vivo experiment the rate of tumor growth in mice (untreated) was sharp from 4th day onward and their mean life span was 20 ± 3 days (Table 1). Tumor growth was found to be inhibited after treatment with CHA, bleomycin and in the combination of treatment as shown in Fig 1. Maximum tumor growth inhibition was observed with combination treatments where the mean life span of tumor bearing mice was 34 ± 4 days (Table 1). Similar enhancement activity of bleomycin has been observed when the latter was encapsulated with liposome (Sur et al., 1984). Correlation between survival of tumor bearing mice and cell growth inhibition are also observed in this combination treatment (Fig. 1 and 2).

The measurement of hematological parameters has shown that bleomycin at dose 0.3mg kg⁻¹ alone is not so effective in restoring the hematological parameters to normal. But enhanced improvement in the hematological parameters are observed in tumor bearing mice treated with bleomycin in combination with CHA (25mg kg⁻¹) (Table 2). The combination treatment thus serves the purpose of increasing survival time with minimal host toxicity. The data obtained for survival time, hematological parameters and ALP activity of tumor bearing mice treated with bleomycin in combination with CHA suggested to conduct this combination treatment with different treatment schedules against different cell lines, which may bring important results in the cancer chemotherapy.

References

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