COMPARATIVE STUDY OF ANTINEOPLASTIC ACTIVITY OF SOME ALIPHATIC AND AROMATIC HYDROXAMIC ACIDS AGAINST EHRLICH ASCITES CARCINOMA (EAC) IN MICE

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SUMMARY: Swiss mice inoculated with Ehrlich ascites carcinoma (EAC) cells were treated with chloroaceto-(CHA), aceto-(AHA), benzo-(BHA) and salicyl-(SHA) hydroxamic acids and also with clinically used anticancer agent hydroxyurea-(HU). Different biochemical (lipid peroxidation in serum, alkaline phosphatase activity) and haematological parameters as well as transplantability of treated EAC cells and enhanced peritoneal macrophages were studied.

Among the hydroxamic acids CHA reduced the tumour weight and enhanced the longivity of tumour bearing mice similar to HU. The antitumour activity of hydroxamic acids were found to be decreased as HU>CHA>SHA>BHA>AHA. Transplantability of EAC cells was reduced by these compounds. Deviated haematological parameters and serum alkaline phosphatase activity in tumour bearing mice were found to be significantly recovered towards normal after treatment with these compounds. However, CHA treatment had recovered these parameters more effectively.

Key Words: Antineoplastic Activity, Ehrlich Ascites Carcinoma.

INTRODUCTION

Hydroxyurea (HU) containing the functional group (-CONHOH) of hydroxamic acid, is a well known anticancer drug (1-4). It inhibits the DNA synthesis by imparing the activity of enzyme ribonucleotide reductase (3-6). Though it is clinically used as anticancer agent, it perturbs the haematological parameters and depresses the bone marrow (7). Subsequently anticancer properties of some aliphatic and aromatic hydroxamic acids (such as AHA, BHA, SHA etc.) have been studied (8,9). Recently it has been reported that CHA possesses antitumour properties and inhibits the growth of EAC cells by imparing DNA and protein synthesis without altering the haematological parameters (10). No such studies have yet been done with AHA, BHA and SHA. This paper reports the comparative study of antineoplastic activities and host toxic effects of hydroxamic acids (CHA, AHA, BHA, SHA) and HU. In addition peritoneal macrophages and lipid peroxida-

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Table 1: Effect of CHA, AHA, BHA and SHA on tumour cell growth inhibition (*in vivo*) and animal life span.

Experiment	No of tumour cells x10^7/mouse on day 5 after tumour inoculation	% of cell growth inhibition	MST	% increase of life span (ILS)
Control (0.9% saline)	1.36 ± 0.21	-	18	-
CHA (100 mg/kg)	0.25 ± 0.05**	81.61	28	55
CHA (25 mg/kg)	0.58 ± 0.09**	57.35	24	33
AHA (100 mg/kg)	0.81 ± 0.03**	41.17	24	33
AHA (25 mg/kg)	0.91 ± 0.08*	30.88	20	11
BHA (100 mg/kg)	0.66 ± 0.09**	51.47	25	38
BHA (25 mg/kg)	0.83 ± 0.05*	38.39	22	22
SHA (100 mg/kg)	0.44 ± 0.08**	67.64	26	44
SHA (25 mg/kg)	0.72 ± 0.04*	47.05	23	27
HU (100 mg/kg)	0.23 ± 0.05**	82.72	29	61
HU (25mg/kg)	0.42 ± 0.03**	69.11	26	44

Swiss A mice were inoculated with EAC cells/mouse (i.p.) on day 0.Treatment with CHA, AHA, BHA, SHA and HU started 24 hours after tumour transplantation and continued for 4 days.On day 5, animals were sacrificed and viable intraperitoneal cells were counted (trypan blue test). Number of mice in each experiment was 6. Results were mean ± SEM. *P<0.01, **P<0.001 when compared with control.

tion in normal mice after treatment with these hydroxamic acids have been presented here. Transplantability of hydroxamic acid treated EAC cells has also been observed.

MATERIALS AND METHODS

Synthesis of the compounds

The compounds CHA, AHA, BHA and SHA were synthesized by the interaction of respective ethyl ester of the acids with the free hydroxylamine according to the usual procedure (11). The purities of these hydroxamic acids were confirmed by elemental analysis and melting points. The reagents and chemicals were purchased from Sigma Chemicals Co (USA) and also from BDH Ltd. (Dugenham U.K.).

For intraperitoneal (i.p) therapeutic treatment of animals with hydroxamic acids, 0.9% normal saline was used as vehicle.

Animals

Adult male Swiss A mice (20-25 gm) were used throughout the study. They were housed in the standard microlon boxes and were given standard mouse pellet, Hindustan Lever, Bombay, India and water *ad libitum.*

Tumour cells

Ehrlich ascites carcinoma (EAC) cells were obtained through the courtesy of Chittaranjan National Cancer Research Center, Calcutta, India. EAC cells were maintained by weekly i.p. transplantation of 10^5 cells/mouse.

Inhibition of tumour growth

1-2x10^5 EAC cells were inoculated into 11 groups of mice (6 in each) on day 0. Mice were treated from day 1 with CHA, AHA, BHA, SHA and HU at dose 100 mg/kg i.p. and 25 mg/kg i.p. The control group was treated with the vehicle, 0.9% saline. The treatment was continued for 4 days and on day 5

Table 2: Bioassay of intraperitoneal EAC cells surviving treatment with CHA, AHA, BHA, SHA and HU.

Experiment	No of tumour cells $x10^7$/mouse on day 5 after inoculation with drug treated EAC cells	% of cell growth
Control (0.9% saline)	1.63 ± 0.20	-
CHA (100 mg/kg)	0.66 ± 0.08**	40.5 (59.50)
AHA (100 mg/kg)	1.02 ± 0.12	62.6 (37.42)
BHA (100 mg/kg)	0.91 ± 0.09	55.8 (44.20)
SHA (100 mg/kg)	0.82 ± 0.07	50.3 (49.70)
HU (100 mg/kg)	0.73 ± 0.06**	44.8 (55.21)

Swiss A mice were inoculated with EAC cells/mouse (i.p.) on day 0. On day 3, mice were treated with CHA,AHA,BHA,SHA and HU at dose 100 mg/kg i.p.EAC cells were collected 24 hours after treatment and reinocultated into fresh mice. On day 5, after tumour (treated) transplantation viable i.p. cells were counted. Number of mice in each group was 4. Results were mean ± SEM. The number shown in the parentheses are the percent of cell growth inhibition. **P<0.001 when compared with control.

after tumour transplantation, animals were sacrificed. Tumour cells were collected by repeated intraperitoneal washings with 0.9% saline. The viable tumour cells were counted (trypan blue test) with a haemocytometer. Total number of viable cells per animal of the treated groups was compared with those of control group.

Survival time

Animals were inoculated intraperitoneally with 1-$2x10^5$ EAC cells/mouse on day 0 and treatment with hydroxamic acids started 24 hours after inoculation, at dose 100 gm/kg/day i.p., and 25 mg/kg/day i.p. The control group was treated with the same volume of saline (0.9%). Treatment was continued for 10 days. Mean survival time (MST) for each group containing 6 mice was noted. Survival time of treated group was compared with those of control group (C) using the following calculation:

$$\text{Per cent increase of life span (\%ILS)} = \frac{\text{MST of treated group}}{\text{MST of control group}} \times 100 - 100$$

Tumour weight was obtained from the weight of mouse on each day from the day of inoculation.

Bioassay of EAC cells surviving *in vivo* treatment

The procedure was a modification of the method of Fernandes and Klubes (12). On day 3 after i.p. inoculation with 1-$2x10^5$ EAC cells, mice received either (a) no drug treatment or (b) CHA (100 mg/kg i.p.), (c) AHA (100 mg/kg i.p.), (d) BHA (100 mg/kg i.p.), (e) SHA (100 mg/kg i.p.) and (f) HU (100 mg/kg i.p.). Four mice were used in each group. 24 hours later, tumour cells from the mice in each group were harvested in cold saline (0.9%), pooled, centrifuged and reinoculated (1-$2x10^5$/mouse) i.p. into five groups containing 4 mice/groups. On day 5, animals from each group were sacrificed and viable tumour cell counts/mouse were performed.

Effect of normal peritoneal cells

Peritoneal cells were counted according to the procedure as described earlier (13). Five groups of normal mice (n=6) were treated with CHA, AHA, BHA, SHA and HU at dose 100 mg/kg i.p. for three consecutive days. The untreated 6th group was used as control. Total peritoneal exudate cells and number of macrophages (stained with 1% neutral red) were counted after 24 hours of treatment and compared with the control.

Measurement of lipid peroxidation by thiobarbituric acid in serum

Thiobarbituric acid reactive substance (TBARS) released in serum was quantitated as follows: serum was isolated from six groups of mice (4 in each). Five groups were treated with CHA, AHA, BHA, SHA and HU at dose 100 mg/kg. i.p. for 4 consecutive days and the 6th group kept untreated (control).

Table 3: Effect of CHA, AHA, BHA, SHA and HU on haematological parameters in tumour bearing mice.

Experiment	Hb gm/dl	RBC ($x10^9$/ml)	WBC ($X10^6$/ml)	Lymphocyte %	Neutrophil %	Monocyte %
Normal mice (control)	14.50 ± 0.54	8.33 ± 0.45	7.00 ± 0.27	70.00 ± 1.80	28.00 ± 2.70	2.00 ± 0.54
EAC bearing mice	9.00 ± 0.88**	4.53 ± 0.46**	32.00 ± 2.40**	40.00 ± 2.00**	56.00 ± 4.00**	1.30 ± 0.27
CHA (100 mg/kg)	13.33 ± 0.25	6.60 ± 0.98	9.00 ± 1.60	52.00 ± 3.00*	46.00 ± 2.05**	2.00 ± 0.28
AHA (100 mg/kg)	10.00 ± 0.70**	5.49 ± 0.76**	17.33 ± 0.60**	48.00 ± 2.05**	49.00 ± 2.50**	3.00 ± 0.42
BHA (100 mg/kg)	8.00 ± 0.25**	4.00 ± 0.14**	18.00 ± 1.19**	42.00 ± 1.7**	55.00 ± 2.94**	3.00 ± 0.06
SHA (100 mg/kg)	10.20 ± 0.59**	5.00 ± 0.34**	14.00 ± 1.6**	50.00 ± 1.30**	46.00 ± 2.8**	2.00 ± 0.5
HU (100 mg/kg)	10.50 ± 0.28**	5.20 ± 0.34**	10.67 ± 1.00**	50.00 ± 2.00**	47.50 ± 2.50**	2.00 ± 0.29

Six groups of Swiss A mice were inoculated with $2x10^5$ EAC cells/mouse (i.p.) on day 0. Treatment with CHA, AHA,BHA,SHA and HU at dose 100 mg/kg i.p. started 24 hours after tumour transplantation and continued for 10 days.The haematological parameters were assessed on day 12 and compared with those of normal group (without tumour or treatment). Number of mice in each group was 4. Results were mean ± SEM. *P < 0.02, **P<0.001 when compared control.

100 m l serum was incubated in 1 ml of 50 m mol phosphate buffer (pH 7) at 37°C for 1 hour. Reaction was stopped by adding 2.5 ml of 20% trichloroacetic acid and 1 ml of 0.67% aqueous solution of thiobarbituric acid (TBA). The pink colour developed (after heating for 10 minutes in a boiling water bath) was measured at 532 nm (14).

Haematological studies

Haemoglobin WBC, RBC and differential counts were estimated (15) in blood from different groups of tumour bearing mice following CHA (100 mg/kg i.p.), AHA (100 mg/kg i.p.), BHA (100 mg/kg i.p.), SHA (100 mg/kg i.p.) and HU (100 mg/kg i.p.) treatment and compared with normal mice without tumour.

Alkaline phosphatase (ALKP) activity

ALKP activity in the serum of normal and tumour bearing mice treated with CHA (100 mg/kg i.p.), AHA (100 mg/kg i.p.), BHA (100 mg/kg i.p.), SHA (100 mg/kg i.p.) and HU (100 mg/kg i.p.) for 10 days was assessed on day 12. The ALKP activity was measured (16) using paranitrophenyl phosphate (PNPP) as substrate, in glycine sodium hydroxide buffer (pH-10). Absorbance was measured at 410 nm with a spectrophotometer. Unit of the enzyme activity is μ mol of PNPP hydrolyzed/min/ml serum.

Statistical analysis

The students t-test was used for the statistical analysis of the results. P values <0.05 were considered significant.

RESULTS

Effect of CHA, AHA, BHA, SHA and HU on growth of EAC cells on day 5 after tumour transplantation is shown in Table 1. Treatment with CHA (100 mg/kg i.p.) resulted in a significant tumour growth inhibition (p<0.001) as evident from 81% reduction of tumour cells, was found to be 82% for HU (100 mg/kg i.p.) treatment. AHA, BHA and SHA at dose 100 mg/kg i.p. inhibited the growth of EAC cells by 41%, 51% and 67% respectively. Significant cell growth inhibition was also observed with all of these hydroxamic acids and HU at dose 25 mg/kg. i.p.

Survival time was found to be increased by reducing tumour weight (Figure 1) after treatment with these compounds. Mean survival time was found to be 29 days (% ILS 61) for the mice treated with HU (100 mg/kg i.p.), which was 28 days (% ILS 55) following CHA (100 mg/kg i.p.), treatment. AHA, BHA, and SHA, treatment at dose 100 mg/kg i.p. showed % ILS of 24, 25 and 26 days respectively. But at dose 25 mg/kg. i.p. hydroxamic acids treatment produced no significant reduction of % ILS (Table 1).

The effect of hydroxamic acids and HU (at dose 100 mg/kg. i.p. single treatment) on transplantability of tumour cells were shown by the reduction of intraperi-

Table 4: Effect of CHA, AHA, BHA, SHA and HU on alkaline phosphatase (ALKP) activity in the serum of tumour bearing mice.

Experiment	ALKP activity $x10^{-3}$ μmol PNPP hydrolyzed/min/ml serum
Normal mice (control)	14.74 ± 0.84
EAC bearing mice	5.86 ± 0.52**
CHA (100 mg/kg)	10.98 ± 0.99*
AHA (100 mg/kg)	7.15 ± 0.42**
BHA (100 mg/kg)	7.43 ± 0.28**
SHA (100 mg/kg)	7.80 ± 0.38**
HU (100 mg/kg)	9.10 ± 0.29**

Six groups of Swiss mice were inoculated with $1x10^5$ EAC cells /mouse (i.p.) on day 0. Treatment with CHA, AHA, BHA, SHA and HU at dose 100 mg / kg i.p. started 24 hours after inoculation and continued for 10 days.The ALKP activity was assessed on day 12 and compared with those of normal group.Number of mice per group was 4. Results were mean ± SEM. *P<0.05, **P<0.001 when compared with control.

toneal tumour burden in mice inoculated with drug treated EAC cells with respect to control. 59.5% reduction of tumour cells burden was observed with CHA whereas the reduction of i.p. tumour burden with HU treated EAC cells was 55%. AHA, BHA and SHA (at dose 100 mg/kg i.p. single treatment) reduced the peritoneal tumour cell burden by 37%, 44% and 49% respectively (Table 2).

The average number of peritoneal exudate cells per normal mouse was found to be $(12.6\pm0.41) \times 10^6$ of which macrophage count was $(3.3\pm0.26) \times 10^6$. Treatment with CHA (100 mg/kg i.p.) and SHA (100 mg/kg i.p.) for three consecutive days resulted in two fold increase in number of macrophages in normal mice. BHA (100 mg/kg i.p.) also enhanced the number of peritoneal macrophages significantly ($p<0.01$). But AHA (100 mg/kg i.p.) and HU (100 mg/kg i.p.) treatment did not show any effect on such enhancement (Figure 2).

Extent of lipid peroxidation was monitored by estimating TBARS in serum of CHA, AHA, BHA, SHA, and HU treated mice TBARS was not enhanced significantly by these compounds.

Haematological parameters (Table 3) of tumour bearing mice on day 12 were found to be significantly altered from those of normal groups. The total WBC count was found to be increased with a reduction of

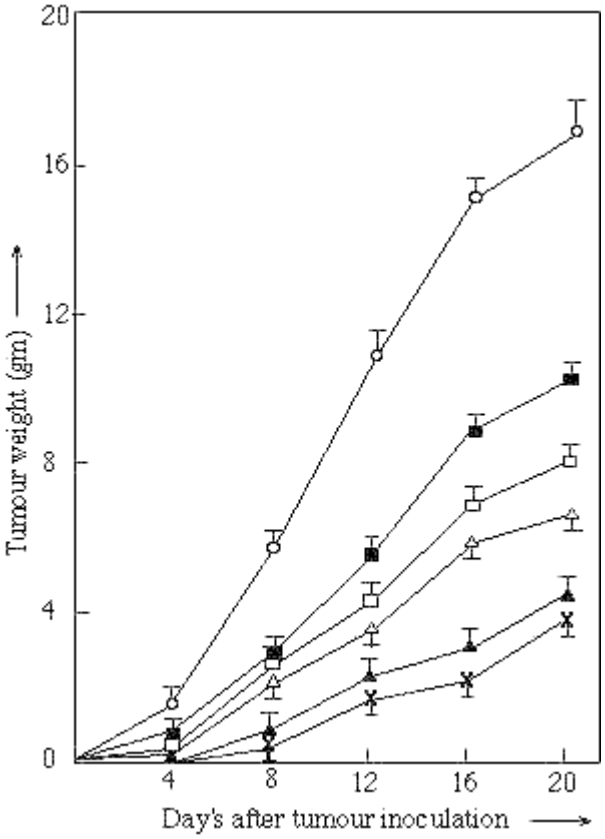

Figure 1: Effect of CHA, AHA, BHA, SHA and HU treatment on tumour weight in mice control -O-O-; AHA (100 mg/kg i.p.) -■-■-; BHA (100 mg/kg i.p.)-□-□-; SHA (100 mg/kg i.p.) -△-△-; CHA (100 mg/kg i.p.)-▲-▲-; HU (100 mg/kg i.p.) -X-X- Number of mice in each group was 8. Results were mean ± SEM.

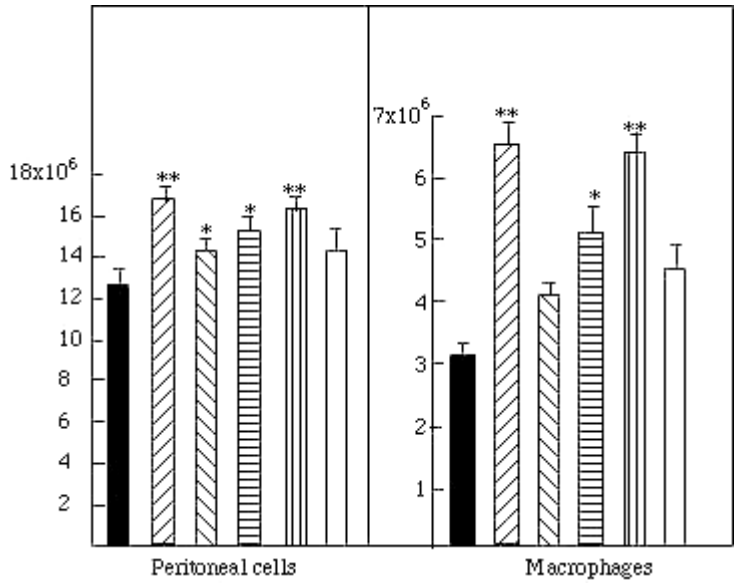

Figure 2: Effect of CHA, AHA, BHA, SHA and HU treatment on enhancement of peritoneal cells in mice. Control - ■; CHA (100 mg/kg i.p.) - ▨; AHA (100 mg/kg i.p.) - ▧; BHA (100 mg/kg i.p.) - ▤; SHA (100 mg/kg i.p.) - ▥; HU (100 mg/kg i.p.) - □. Number of mice in each group was 6. Results were mean ±SEM. *P < 0.02 – 0.01; ** P < 0.001 when compared with control.

haemoglobin content of RBC. The total number of RBC showed a modest change. In differential count of WBC, the percent of neutrophils increased while the lymphocyte count decreased. At the same time interval only CHA (100 mg/kg i.p.) treatment could restore all the altered haematological parameters towards normal value. HU (100 mg/kg i.p.) treatment also recovered these depleted parameters towards normal though CHA treatment was found to be more effective. AHA and SHA at dose 100 mg/kg i.p. altered these parameters to some extent. BHA (100 mg/kg i.p.) treatment showed toxic effect reflected by depletion of haemoglobin and RBC values, which could not be recovered.

Growth of EAC tumour decreased ALKP activity in serum of tumour bearing mice. Appreciable improvements were observed on day 12 following CHA (100 mg/kg i.p.) treatment, which showed 74% activity with respect to normal value. In HU (100 mg/kg i.p.) treated tumour bearing mice activity was 61%, BHA and SHA (at dose 100 mg/kg i.p.) also altered the depleted ALKP activity very modestly, which showed 50% and 52% activity respectively (Table 4).

DISCUSSION

From the above results it has been concluded that CHA, AHA, BHA and SHA inhibit the growth of EAC cells (*in vivo*) consecutively reduce tumour weight and enhance the life span of tumour bearing mice significantly. CHA treatment increases the life span of tumour bearing mice similarly as HU, showing maximum antitumour activity. The antitumour activity of these compounds can be arranged in decreasing order as HU>CHA>SHA>BHA>AHA. This variation is probably due to the different molecular structure of the compounds. Hydroxamic acid functional group (-CONHOH) is responsible for antitumour property of HU (17,18). The replacement of $-NH_2$ group of HU by methyl group leads to the decrease in antitumour activity, whereas the aromatic group substitution favours the activity (9). In our experiments similar effects have been noticed with SHA and BHA. On the other hand, replacement of $-NH_2$ group with chlorosubstituted ($-CH_2Cl$) group retains the antitumour activity against EAC cells, which is almost similar to HU. AHA, BHA and SHA inhibit the growth of EAC cells by impairing the DNA synthesis (9)

only but CHA inhibits both DNA and protein synthesis (10). The bioassay experiment shows reduction of transplantability of EAC cells previously treated (i.p.) with CHA, AHA, BHA, SHA and HU, indicating loss of viability of the treated cells. CHA treatment shows the maximum effect for the loss of transplantability of EAC cells, while the other compounds show the modest effect in the order of CHA>HU>SHA>BHA>AHA.

To evaluate whether CHA, AHA, BHA, SHA and HU treatments indirectly inhibit tumour cell growth, the effects of i.p. treatment of these compounds on the peritoneal exudate cells of normal mice are observed. In our experimental model each normal mouse contains about $12x10^6$ intraperitoneal cells 25% of which are macrophages. CHA and SHA treatments are found to enhance the macrophage counts very significantly. Enhancement and activation of macrophages might produce some cytokine products, such as tumour necrosis factor (TNF), interleukins etc., inside the peritoneal cavity, which in turn may be as well responsible in killing the tumour cells (19).

Free radicals could serve as powerful agents to kill tumour cells (20). The presence of TBARS in serum is due to the generation of free radicals in the system. No such TBARS is observed in serum after treatment with CHA, AHA, BHA, SHA and HU.

Perturbation of haematological parameters in tumour bearing animals is partly due to the toxic effects produced in them. In addition, myelosuppression in cancer chemotherapy is a common phenomenon, which is responsible for poor prognosis (21). CHA treatment restores the haematological parameters more effectively than HU and other hydroxamic acids in the following order CHA>HU>SHA>AHA>BHA. BHA treatment shows host toxicity as reflected in haemoglobin and RBC profile. Depletion of ALKP activity in tumour bearing mice is also found to be restored by these compounds in the order CHA>HU>SHA>BHA> AHA.

In the light above observations, CHA can be considered as the most effective antitumour agent amongst the hydroxamic acids studied and is comparable with HU regarding cell growth inhibition and survival time of tumour bearing mice. However, it is necessary that the antitumour activity of CHA should be carried out against different tumour cell lines which may bring promising results in cancer chemotherapy.

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