
Research Paper

The Sciences 1 (5): 339-342
September-October, 2001

The Sciences (ISSN 1608-8689)
is an International Journal
serving the International
community of Medical
Scientists

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Antineoplastic Activity of Cyanohydroxamic Acid (Sodium Salt) Against Ehrlich Ascites Carcinoma in Mice

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Antitumor activity of sodium salt of cyanohydroxamic acid (CNHA) has been evaluated against Ehrlich ascites carcinoma (EAC) in mice. CNHA inhibited the growth of EAC cells and enhance the longitivity of tumor bearing mice significantly. Hematological parameters and alkaline phosphatase (ALP) activity in tumor bearing mice are found to be significantly altered but CNHA treatment recovered those parameters towards normal values. High mortality rate of brine shrimp nouplii was obtained with CNHA exhibiting pronounced cytotoxic effect.

Key words: CNHA, Antitumor activity, hematology, brine shrimp nouplii

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Introduction

Hydroxyurea (HU) containing the functional group (-CONHOH) of hydroxamic acid, is a well known anticancer drug (Donehower, 1990; Gora and Robak, 1995; Coutinho *et al.*, 1996; Nand *et al.*, 1996). It inhibits the DNA synthesis by impairing the activity of enzyme ribonucleotide reductase (Nand *et al.*, 1996; Santarossa *et al.*, 1995). Though it is clinically used as an anticancer agent, it perturbs the hematological parameters and depresses the bone marrow (Bruce and Kennedy, 1970). Subsequently anticancer properties of some aliphatic and aromatic hydroxamic acids such as aceto (AHA), benzo (BHA), salicylic (SHA) and chloroaceto (CHA) hydroxamic acid have been investigated (Moore, 1969; Elford, 1979; Sur *et al.*, 1997). AHA, BHA and SHA inhibits the DNA synthesis by impairing the activity of ribonucleotide reductase also (Elford *et al.*, 1979) and CHA inhibited the growth of EAC cells by inhibiting the DNA and protein synthesis (Sur *et al.*, 1997). No such research works have yet been done with cyanohydroxamic acid (CNHA). This paper reports the antineoplastic activity and host toxic effect of CNHA. In addition cytotoxic effect of CNHA have been recorded.

Materials and Methods

Synthesis of sodium salt of cyanohydroxamic acid (CNHA):

The (CNCH₂-C-NHOH) compound CNHA was synthesized by interaction of ethyl ester of cyanoacetate with free hydroxylamine according to the procedure described by Jones and Wemer (1917). The compound was isolated as sodium salt. It was purified by repeated wash with alcohol (C₂H₅OH) and confirmed by functional group test and I.R. spectra. The reagents and chemicals were purchased from Sigma Chemicals Co. (USA) and also from BDH Ltd. (Dugenhams UK).

Animals: Adult Swiss male mice (20-25gm) were obtained from International Center for Diarrhoea Disease Research, Bangladesh (ICDDR'B). Animals were fed with standard mouse pellet collected from ICDDR'B and water was given ad libitum.

Tumor cells: Ehrlich ascites carcinoma (EAC) cells were obtained from Indian Institute for Chemical Biology, Calcutta-700032, West Bengal, India and were maintained by weekly intraperitoneal (i.p.) inoculation of 10⁵ cells/mouse in the laboratory.

LD₅₀ determination: To get the LD₅₀ dose of CNHA, similar experiment was performed with mice as reported by Litehfield and Wilcoxon (1949). The drug solution in water was administered in different doses (i.p.) in male swiss mice. LD₅₀ was evaluated by recording mortality upto 24 hours.

Cell growth inhibition: *In vivo* tumor cell growth inhibition (Sur and Ganguly, 1994) were carried out with CNHA and HU. Animals (6 in each group) were inoculated with 2 x 10⁶ cells mouse⁻¹. Treatment with CNHA at dose 100, 50, 25mg kg⁻¹ and HU (100mg kg⁻¹) started after 24 hrs of inoculation. The control group was treated with vehicle (normal saline). All the treatments were continued for 4 consecutive days. Animals were sacrificed on day 5 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 CNHA and HU were carried out under similar experimental

conditions as stated in previous experiment for 10 days using the dose for CNHA 100, 50 and 25 and for HU 100mg kg⁻¹, respectively. Mean survival time (MST) for each group containing 6 mice was noted. Survival time of treated groups were compared with those of control group with the following calculation (Sur *et al.*, 1984).

$$\% \text{ increase in life span over control} = \frac{\text{MST of treated group}}{\text{MST of control group}} \times 100 - 100$$

$$\text{where, MST} = \frac{\sum \text{Survival time (days) of each mouse in a group}}{\text{Total no. of mice}}$$

Life span with respect to control (T/C %) = (MST of treated group/ MST of control group)x100

Hematological studies: In order to detect the influence of CNHA (100mg kg⁻¹) on hematological status of normal and EAC bearing mice, comparison were made among four groups (n = 4) of mice on the 12th day, after tumor transplantation (Khanam *et al.*, 1997). The four groups comprised (1) normal mice served as control, (2) mice treated with CNHA mg kg⁻¹, (3) EAC bearing mice and (4) EAC bearing mice treated with CNHA(100mg kg⁻¹). Treatment was continued for 10 consecutive days. Blood was collected from individual mice for 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 (Ruisa and Swaroop, 1988) using cell diluting fluids and a hemocytometer. The differential count was carried out with Wright stain.

Alkaline phosphatase activity (ALP): ALP activity in the serum of EAC bearing mice of all such groups [treated with CNHA and untreated EAC bearing mice (n=4)] were assayed with the following treatments; (a) normal untreated mice, (b) tumor bearing mice and (c) tumor bearing mice treated with CNHA (100mg kg⁻¹, 10 days). Blood was collected from individual mouse in a group and assayed for ALP activity by the standard method reported by Michell (1970) on 12th day after tumor inoculation.

Brine shrimp lethality Bioassay: Twelve vials were taken (two vials for each concentration). 5ml sea water was given to each of the vials. 5, 10, 20, 40 and 80gm ml⁻¹, respectively, solutions of CNHA were transferred to 10 vials and 2 vials were used as control. With the help of Pasteur pipette 10 living shrimps were inoculated into each of the vials. After 24 hrs., vials were observed and number of survival noplili in each vials were counted (Attaur and Thomsen, 1999; Meye, 1982).

Statistical analysis: Significance of the experiments were statistically evaluated (Ghosh, 1984) by students "t" test and significance was assigned when p < 0.05.

Result

LD₅₀ value of CNHA was found to be 775mg kg⁻¹. Effect of CNHA on growth of EAC cells on day 5 after tumor transplantation has shown in Table 1. Treatment with CNHA (100mg kg⁻¹) resulted in a significant tumor growth inhibition (P < 0.001), as evident from 72.44% reduction of tumor cells, which was found to be 82% for HU (100mg kg⁻¹) treatment. Significant cell growth inhibition was also observed with CNHA at dose 50 and 25mg kg⁻¹ which were 65.90 and 60.68%, respectively.

As shown in Table 2 the mean survival time (MST) for the

Table 1: Effect of CNHA (Cyanohydroxamic acid) on EAC (Ehrlich ascites carcinoma) cell growth (*in vivo*)

Experiment	Dose mg kg ⁻¹	No. of tumor cell 10 ⁷ /mouse on day 5 after tumor inoculation	% of cell growth inhibition
Control (0.9% Saline)	-----	9.39 ± 0.54	-----
CNHA	100	2.59 ± 0.34**	72.44
CNHA	50	3.2 ± 0.34**	65.92
CNHA	25	3.69 ± 0.37**	60.70
HU	100	1.69 ± 0.27**	82.72

Results shown are mean values ± SEM. No. of mice each group is 8 **P < 0.001.

Table 2: Survival of EAC bearing mice following CNHA treatment

Treatment (n = 8)	Dose (mg kg ⁻¹ day ⁻¹)	MST (days)	T/C %	Increase in life span over control (%)
Control (saline)	-	34 ± 1.7	100	-
CNHA	100	51 ± 1.5**	150	50
CNHA	50	49 ± 1.6**	144	44
CNHA	25	40 ± 1.5*	117	17
HU	100	55 ± 1.6**	161	61

**P < 0.001, *P < 0.05

Table 3: Effect of CNHA (100 mg kg⁻¹ 10 days) treatment on hematological parameters in normal and tumor bearing mice

Experiment	Hb (gm dl ⁻¹)	RBCx 10 ⁹ ml ⁻¹	WBCx 10 ⁶ ml ⁻¹	Lymphocyte (%)	Neutrophil (%)	Monocyte (%)
Normal mice (0.9% saline) control	12.0 ± 0.97	7.98 ± 0.47	5 ± 0.38	66 ± 2.40	25 ± 2.227	4 ± 0.38
EAC bearing mice	7.12 ± 0.48*	3.82 ± 0.31**	25 ± 0.46**	38 ± 1.73**	54 ± 2.26**	1.3 ± 0.38
EAC bearing mice + CNHA (100mg kg ⁻¹)	10.1 ± 0.46*	5.87 ± 0.39**	10 ± 0.37**	59 ± 1.92	36 ± 2.7	3 ± 0.25
Normal mice + CNHA (100mg kg ⁻¹)	11.62 ± 0.12	6.1 ± 0.29	9.24 ± 0.40	50 ± 2**	23 ± 0.20	3.35 ± 0.35

Hematological parameters were studied on day 12 after tumor transplantation. Results were mean ± SEM n = 4, **P < 0.001, *P < 0.05-0.01.

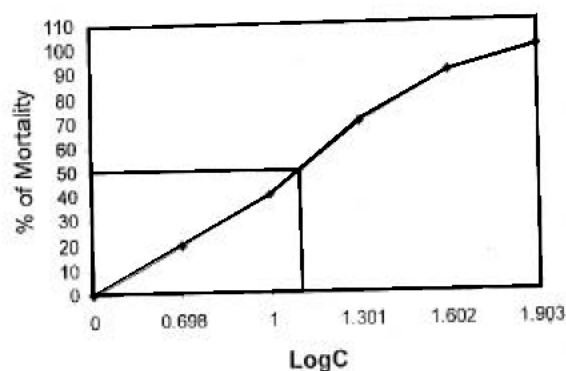


Fig. 1: Effect of CNHA on brine shrimp mortality C = Concentration of CNHA, CNHA = Cyanohydroxamic acid

control group was found to be 34 days, while it was 51 days for the group treated with CNHA (100mg kg⁻¹, 10days), T/C% was 151. At doses 50mg kg⁻¹, 10days and 25mg kg⁻¹, 10 days, the CNHA showed the MST 49 and 40 days and T/C% 144 and 117, respectively. HU at dose 100 mg kg⁻¹, treatment resulted MST 55 and T/C% 161.

Hematological parameters (Table 3) of tumor bearing mice on day 12 were found to be significantly (P < 0.001, 0.05) altered from normal group. The total WBC count was found to be increased with a reduction of hemoglobin. The total number of

RBC was also found to be decreased and the differential count of WBC, the % increase of neutrophils increased while the differential count decreased. At the same time interval CNHA (100mg kg⁻¹, 10 days) treatment could restore hemoglobin and RBC values to the normal like HU (100mg kg⁻¹, 10 days) treatment. The increased WBC counts and differential counts were also found to be altered towards normal following CNHA treatment.

In the case of normal mice, CNHA treatment did not showed any significant change on hematological parameters where as HU (100mg kg⁻¹, 10 days) treatment resulted myelosuppressive effect in this respect. Growth of EAC cells in mice showed a depletion of ALP activity in serum. Significant recovery of the enzyme activity was found in the serum of tumor bearing mice following CNHA treatment. To find out the effect of CNHA on the mortality of brine shrimp noplili median lethal concentration of brine shrimp (LC₅₀) was measured and it was found to be 12.74 μg ml⁻¹. The plot of percent mortality versus log of CNHA concentration showed an approximate linear correlation between them (Fig. 1).

Discussion

From the results it has been found that CNHA inhibits the growth of EAC cells *in vivo* and consequently increases the life span of tumor bearing mice. It is known that HU and other hydroxamic acids in general act as ribonucleotide reductase inhibitors (Elford *et al.*, 1979). Young *et al.*, 1967 have shown that replacement of -NH₂ group by methyl group (-CH₃) in HU leads to decrease the antitumor activity. In this experiment replacement with cyano group (-CN) retains the antitumor activity like HU. Antitumor activity of CNHA may be due to the

presence of cytotoxic effect of it. Cytotoxic effect has been confirmed from low value of LC₅₀ (Fig. 1).

Perturbation of hematological parameters in tumor bearing mice are partly responsible for the toxic effects produced in them. In addition myelosuppression in cancer chemotherapy is a common phenomenon which is responsible for poor prognosis (Donehower, 1990). CNHA inhibits tumor cell growth, enhances the survival of treated mice and restores the hematological parameters. The depletion of ALP activity in tumor bearing mice is also found to be restored by CNHA treatment. The observations described above showed the efficiency of CNHA, with the dose mentioned above, having no adverse side effect on the host. Based on these observations, it can be concluded that CNHA possesses some antitumor properties and further researches can be carried out in an advanced level, in the field of cancer treatment.

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MS received 2nd October, 2001; Accepted 15th October, 2001