Antitumor Activity of a Triazole Derivatives(S_1) Against Ehrlich Ascites Carcinoma (EAC) Bearing Mice

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Abstract

In search of potential anticancer drug candidates, a triazole derivative designated by S_1 has been synthesized and characterized. Its anti-neoplastic activity has been studied against Ehrlich Ascites Carcinoma (EAC) cells in swiss albino mice by monitoring the tumor weight measurement, survival time, tumor cell growth inhibition, haematological characteristics etc. It has been found that the compound, at dose 2.0 mg/kg/day (i.p), significantly decreased the tumor weight and the tumor cell growth rate, and increased the life span of mice as compared to those of EAC bearing mice. The compound also altered the depleted haematological parameters like RBC, WBC including differential counts (e.g. lymphocytes, neutrophils, monocytes etc) of %Hb of EAC bearing mice towards normal. The compound enhanced the number of macrophages in normal mice. The toxic effects of the compound as the host were not very high and the animals recovered gradually towards normal within a few days after treatment. The results suggested that the compound is capable to exhibit significant antitumor property with little adverse affects on the hematological profiles of the host.

Key words: Antineoplastic activity, EAC cell, triazole derivatives, Haematology

Introduction

Cancer continues to represent the largest cause of mortality in the world and claims over six million lives every year (Abdullaev *et al.,* 2000). An extremely promising strategy for cancer prevention today is chemotherapy. For developing chemotherapeutic agents, various compounds like Schiff bases have drawn the attention of many investigators (Jesmin *et al.,* 2008; Molla *et al.,* 2000; Klayman *et al.,* 1983) owing to their potential applications. In some cases these compounds have been proved to possess anti-leukemic4 effects. Now a days 1,2,4-triazoles and their derivatives have been found to be associated with various biological activities such as anticonvulsant (Küçükgüzel *et al.,* 2004), antifungal (Rollas *et al.,* 1993), anticancer (Holla *et al.,* 2003, Bekircan *et al.,* 2005) and antibacterial (Ikizler *et al.,* 1999) properties. Several compounds containing 1,2,4-triazole rings are well known as drugs. For example, fluconazole is used as an antimicrobial drug (Shujuan *et al.,* 2004) while vorozole, letrozole and anastrozole are non-steroidal drugs used for the treatment of cancer (Clemons *et al.,* 2004). Recently some Schiff base derivatives of 1,2,4-triazoles and their reduced derivatives have also been found to possess pharmacological activities (Kahveci *et al.,* 2005; Bekircan *et al.,* 2006). These data prompted us to synthesize a triazole derivative namely 2-(5-mercapto-4-phenyl-4H-[1,2,4] triazole-3-yl]-cyclohexa-1,5-dienol (S_1) and evaluate its antineoplastic activity against Ehrlich Ascites Carcinoma (EAC) cells in swiss albino mice.

Materials and Methods

Chemicals and reagents: All the chemicals and reagents used throughout the investigation were of reagent grade.

Animals: Adult swiss albino male mice (20-25 g) were used for the studies. They were obtained from the International Center for Diarrheal Diseases Research, Bangladesh (ICDDR,B). The animals were fed with standard mouse-pellets (collected from ICDDR,B) and water ad libitum.

Tumor cells: Ehrlich Ascites Carcinoma (EAC) cells were obtained from the Indian Institute for Chemical

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Biology, (IICB), Kolkata, India and were maintained by weekly intraperitoneal (i.p.) inoculation of 14×10^5 cells/mouse in the laboratory.

Ethical clearance: Protocol used in this study for the use of mice as animal model for cancer research was approved by the University Animal Ethical committee.

Determination of median lethal dose (LD_{50}): The LD_{50} value was determined following conventional method (Litchfield *et al* 1949). The test compound was dissolved in distilled water with the help of Tween-80 and injected intraperitoneally to six groups of mice (each containing six) at different doses (25, 50, 75, 100, 125 and 150 mg/kg). LD_{50} was evaluated by recording mortality after 24 hours.

Cell growth inhibition: In vivo tumor cell growth inhibition was carried out by standard method (Sur *et al* 1994). For this study 5 groups of mice (6 in each group) were used. For therapeutic evaluation 14×10^5 cells/mouse were inoculated into each group of mice on the first day. Treatment was started after 24 hours of tumor inoculation and continued for 5 days. The mice of groups 1 to 3 received the test compound at 1.0 mg/kg, 2.0 mg/kg and 4.0 mg/kg, respectively per day per mouse. In each case, the volume of the test solution injected was 0.1ml/day per mouse. Group 4 received *bleomycin* (0.3 mg/kg, i.p) and finally the mice of group 5 was treated with the vehicle (normal saline and Tween-80) and was considered as negative control. The mice were sacrificed on the 6th day after transplantation and tumor cells were collected by repeated intraperitoneal wash with 0.9% saline. Viable tumor cells per mouse of the treated group were compared with those of control. The cell growth inhibition was calculated by using the formula,

$$\% \text{ Cell growth inhibition} = \left(1- \frac{Tw}{Cw}\right)\times100$$

where, Tw = Mean of number of tumor cells in the treated group of mice and Cw = Mean of number of tumor cells in the control group of mice.

Bioassay of EAC cells (Transplantation ability of EAC cells): The effect of S_1 on transplantability of EAC cells was carried out by the method described in the literature (Fernandes *et al.*, 1979). In this experiment two groups of mice (n=4) were inoculated with 115×10^5 EAC cells. Group 1 was treated with the test compound at the dose of 4.0 mg/kg (i.p.) for five consecutive days and group 2 served as control. On day 7, tumor cells from the mice were harvested in cold (0.9%) saline, pooled, centrifuged and re-inoculated into two fresh groups of mice (n=4) as before. No further treatment was done to these mice. On day 5, they were sacrificed and viable tumor cells count/mouse was performed.

Average tumor weight and survival time: These parameters were measured under similar experimental conditions as stated in the previous experiment. Tumor growth was monitored daily by measuring the change in weight. The host survival time was recorded and expressed as mean survival time in days and percent increase of life span was calculated (Abbot *et al* 1976) as follows:

$$\text{Mean survival time (MST)} = \frac{\sum \text{Survival time (days) of each mouse in a group}}{\text{Total number of mice}}$$

$$\text{Percent increase of life span (ILS) \%} = \left(\frac{\text{MST of treated group}}{\text{MST of control group}} - 1\right)\times 100$$

Haematological studies: The haematological parameters viz WBC, RBC, Hb content, differential counts etc were determined by the standard methods (Hudson *et al* 1989) using cell dilution fluids and haemocytometer. For this purpose, blood was collected from the mouse by tail puncture method. Three groups of mice (with n=4) were taken for doses 1.0, 2.0 and 4.0 mg/kg of the test compound (S_1). Treatment started after 24 hours of tumor transplantation and was continued for 10 consecutive days. On day 12, the blood parameters were assayed and the data were tabulated in Table 1.

For normal mice, four groups (n=4) were taken for the purpose. The blood from the mice of group I was assayed on day 0 (without any treatment). The second and third groups of mice were treated with S_1 at dose 4.0 mg/kg for 5 and 10 consecutive days respectively and analyzed as before. The mice of 4th group were treated with 2.0 mg/kg for 10 consecutive days and assayed on day 25. The data were furnished in Table 2.

Determination of the effect of S_1 on normal Peritoneal cells: The effects of compound on normal peritoneal cells were determined (Meyer *et al* 1982) by counting total peritoneal cells and number of macrophages. Three groups of mice (4 in each) were treated with S_1 at doses 1.0, 2.0,

and 4.0 mg/kg for three consecutive days, the fourth (untreated) group (n=4) served as control. After 24 hours of treatment, each animal was injected with 5 ml of normal saline (0.98%) into the peritoneal cavity and then sacrificed. Intraperitoneal exuded cells and number of macrophages were counted with 1% neutral red by haemocytometer.

Table 1. Effect of S_1 on blood parameters of tumor bearing and normal swiss albino mice on day 12 of tumor inoculation.

Name of Exp.	RBC Cells/ml	WBC Cells/ml	% of Hb gm/dl	% of Lymphocyte	% of Neutrophil	% of Monocyte
Normal mice	$(6.11 \pm 0.40) \times 10^9$	$(10.4 \pm 1.2) \times 10^6$	11.6 ± 1.2	72 ± 0.3	23 ± 0.2	5 ± 0.65
Control (EAC bearing) mice	$(2.32 \pm 0.2) \times 10^9$	$(25.7 \pm 0.4) \times 10^6$	5.0 ± 0.70	51 ± 0.65	36 ± 1.2	13 ± 0.6
EAC+ 1.0 mg/kg	$(2.3 \pm 0.15) \times 10^9$	$(18.0 \pm 0.45) \times 10^{6*}$	$9.4 \pm 0.21^{***}$	47 ± 0.54	38 ± 0.41	15 ± 0.21
EAC+ 2.0 mg/kg	$(4.2 \pm 0.24) \times 10^9$	$(14.0 \pm .41) \times 10^{6*}$	$11.7 \pm 0.36^{***}$	$65 \pm 0.24^{***}$	$31 \pm 0.54^{*}$	$4 \pm 0.18^{***}$
EAC+ 4.0 mg/kg	$(5.1 \pm .21) \times 10^{9***}$	$(12.0 \pm .24) \times 10^{6**}$	$11.\pm 0.41^{***}$	$70 \pm 0.25^{***}$	$28 \pm 0.45^{**}$	$2 \pm 0.54^{***}$

Number of mice in each case was 4; the results were shown as mean ± SEM and compared with control (EAC bearing), where significant values are,*p<0.05,**p<0.01 and***p<0.001.

Table 2. Effects of S_1 on blood parameters in normal mice on days, 0, 5, 10 and 25 at dose 4.0 mg/kg body weight.

Name of Exp.	Day(s)	RBC Cells/ml	WBC Cells/ml	% of Hb	% of Lymphocyte	% of Neutrophil	% of Monocyte
Normal mice	0	$(6.11 \pm 0.40) \times 10^9$	$(10.4 \pm 1.2) \times 10^6$	11.6 ± 1.2	72 ± 0.3	23 ± 0.2	5 ± 0.65
Mice treated with S1	5	$(4.3 \pm 0.71) \times 10^{9**}$	$(6.85 \pm 0.91) \times 10^{6***}$	$5.2 \pm 0.10^{***}$	$62 \pm 2.0^{**}$	$31 \pm 0.97^{**}$	7 ± 0.85
	10	$(5.58 \pm 0.82) \times 10^{9**}$	$(7.12 \pm .21) \times 10^{6**}$	$8.2 \pm 0.19^{***}$	$63 \pm 3.1^{**}$	$33 \pm 1.2^{**}$	4 ± 1.2
	25	$(6.5 \pm 0.54) \times 10^9$	$(8.43 \pm 1.1) \times 10^{6**}$	10.7 ± 01.3	71 ± 2.1	20 ± 0.92	9 ± 1.4

Number of mice in each group was 4. Result were shown as Mean ± SEM and compared with control with normal mice (without treatment) where significant values are*p<0.05,**p<0.01 and***p<0.001

Statistical analysis: The experimental results have been expressed as the mean ± SEM (Standard Error of Mean). Data have been calculated by one way ANOVA followed by Dunnett 't' test using SPSS software of version 10.

Results and Discussion

In vivo tumor cell growth inhibition was observed with S_1 at various doses (1.0- , 2.0- , and 4.0- mg/kg) per mouse per day. Maximum cell growth inhibition (about 82.13%) was found after treatment with the drug at 4.0 mg/kg, bleomycin at dose 0.3 mg/kg on the other hand inhibited the cell growth by 89.57% (Figure 1). As high as 69.06% reduction in EAC cell growth was observed when 6 days treated EAC cells were re-inoculated into fresh mice (viable cells counts were performed on day 5).

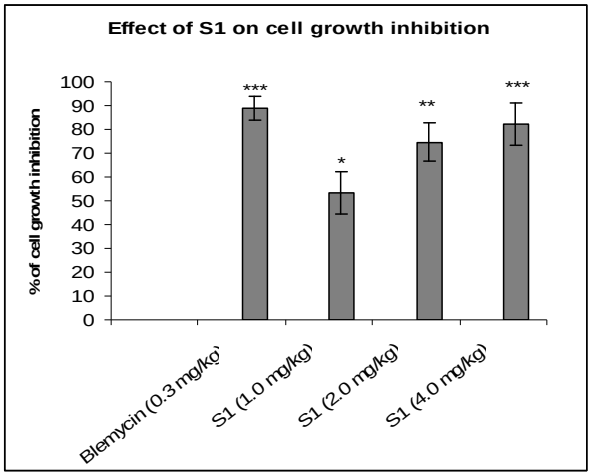

Figure 1. Effect of S_1 on *in-vivo* inhibition of EAC cell growth in mice

Number of mice in each case was 6; the results were shown as mean ± SEM and compared with control, where significant values are *p<0.05, p^{**}<0.01 and *** p<0.001.

The mean survival time (MST) of the untreated tumor bearing mice was 19.83 days. This value increased remarkably due to treatment with S_1. About 74% enhancement of MST was found at 4.0 mg/kg. On the contrary, bleomycin at the dose 0.3 mg/kg, increased this value to 94.15 % (Figure 2).

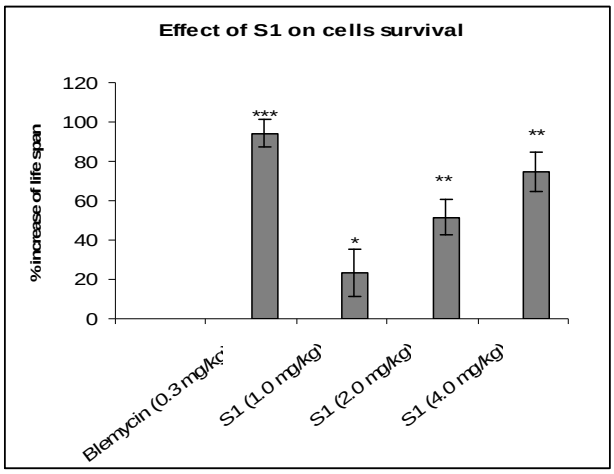

Figure 2. Effect of S_1 on survival time of EAC cell bearing mice

Number of mice in each case was 6; the results were shown as mean ± SEM and compared with control, where significant values are *p<0.05, p^{**}<0.01 and *** p<0.001.

The treatment with compound S_1 also reduced the rate of tumor growth. The reduction of tumor growth was found to be only 28 % at dose 4.0 mg/kg after 20 days as compared to 85.5% without any treatment. With *bleomycin* (0.3 mg/kg), however, the growth reduced to 21% in 20 days (Figure 3). The use of higher dose of the test compound showed greater tumor growth inhibitory activity (data are not presented here).

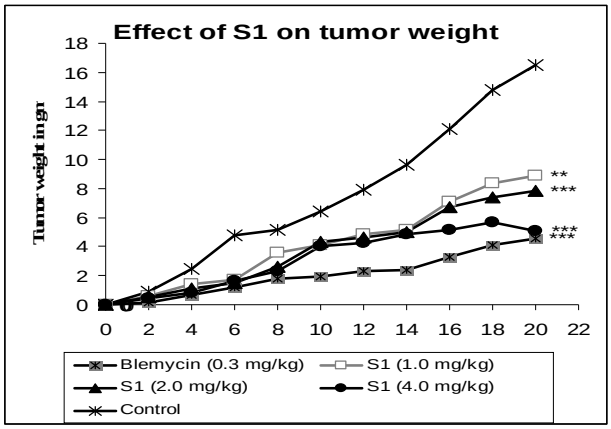

Figure 3. Effect of S_1 on tumor weight in mice

Number of mice in each case weas 6; the results were shown as mean ± SEM and compared with control, where significant values are *p<0.05, p^{**}<0.01 and *** p<0.001.

The haematological parameters of both treated and non-treated mice were examined. In EAC cell bearing mice all the parameters such as haemoglobin, WBC, RBC and differential counts (monocytes, lymphocytes, neutrophil, monocytes) were found to be significantly changed as compared to those of the normal mice. These parameters moved towards normal values when treated with the compound at dose 4.0 mg/kg. In case of parallel treatment of normal mice, these parameters were found to be slightly changed from normal values and after 25 days of treatment, they restored almost towards normal (Table-1, 2). It has been found that the compound at dose 2.0 mg/kg has enhanced both the peritoneal cells and the number of macrophages to some extent in normal mice. The results have been presented in Table 3.

Table 3. Effect of S_1 on the enhancement of normal peritoneal cells in mice

Name of experiment	Dose mg/kg	Macrophages Mean ± SEM	Total peritoneal cells Mean ± SEM
Control (Normal)	-	$(1.67 \pm 0.31) \times 10^6$	$(8.7 \pm 0.40) \times 10^6$
Normal + test compound	1.0	$(3.15 \pm 0.5) \times 10^{6***}$	$(9.42 \pm 0.34) \times 10^{6***}$
	2.0	$(4.35 \pm 0.40) \times 10^{6***}$	$(10.12 \pm 0.44) \times 10^{6***}$
	4.0	$(5.57 \pm 0.55) \times 10^{6***}$	$(11.3 \pm 0.43) \times 10^{6***}$

Number of mice in each case was 6; the results were shown as mean ± SEM and compared with normal mice (without treatment), where significant values are,*p<0.05,**p<0.01 and***p<0.001

The toxicity of this compound was evaluated by measuring LD_{50} values in swiss albino mice. The value was found to be 60 mg/kg.

The results presented above showed that the compound at dose 4.0 mg/kg can inhibit cell growth of tumor bearing mice satisfactorily, reduce tumor growth rate uniquely and increase life span significantly. All

these are considered as very important and promising aspects in justifying the potency in cancer chemotherapy (Price *et al.,* 1958). One of the major problems usually encountered in cancer chemotherapy is myelosuppression followed by anemia (Hoaglad *et al.,* 1982; Fenninger *et al.,* 1954) the reduction of RBC and haemoglobin contents. This is probably owing to the deficiency of iron of haemolytic or myelopathic condition (Barger *et al.,* 1981) treatment of the compound can also reverse back all the depleted haematological parameters towards normal. The toxic effect of the compound on the host was not found very high. In addition, the treatment in normal mice increases the macrophages and peritoneal cells which have also been considered as very vital event in acquiring self destroying activity of the living beings towards cancer cells (Fernandes *et al.,* 1979) enhancement of macrophages might produce some cytokines such as tumor necrosis factors (TNF), interleukins etc inside the peritoneal cavity, which in turn may be responsible for killing of tumor cells (Lee *et al*. 1982). The transplant ability of EAC cells shows their significant reduction of viability after the treatment with S_1. In addition, since LD_{50} value of S_1 was found to be 60 mg/kg as compared to the dose used during treatment, the compound (S_1) can be considered as an effective anticancer agent. However, the findings of the present work at this stage are not so definitive to claim as an achievement in formulating new anticancer drug. Much work should be done with this compound and also with its derivatives using various cell lines and higher animal models in order to prove it as a potential anticancer agent.

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