Original Research Articles

Antineoplastic Activity of the Ethyl Acetate Extracts from Co-cultured Marine and Terrestrial Actinomycetes

ABSTRACT

Treatment of cancer is mainly depending on chemotherapy. A considerable number of antitumor compounds are obtained from microorganisms. Particularly, actinomycetes are the sources of a number of compounds with different biological activities, including antitumor properties. Recent reports by some scientists have revealed a new dimension in producing new bioactive compounds by co-culturing the antibiotic producing actinomycetes. This study was aimed to investigate the effect of co-culturing the antibiotic producing bacteria in liquid broth media among themselves. We used two strains of actinomycetes named ANTS-1 & AIAH-10, which was previously collected from the normal and marine soil of Bangladesh. Antineoplastic activity of co-cultured bacterial metabolites named AIKA-110 was investigated against Ehrlich Ascites Carcinoma (EAC) cells in Swiss Albino mice. AIKA-110 enhanced the mean survival time (MST) of tumor bearing mice at 70.76% and maximum cell growth inhibition was found 74.66% with a dose of 100mg/kg (intraperitoneal). Moreover, hematological parameters of AIKA-110 were found to restore moderately only the high dose. For instance, total RBC count and hemoglobin count was increased but total WBC count was decreased. These findings highlight that the co-culture of microbes triggered to the production of a new compound with promising antineoplastic activities.

Keywords: Co-culture, Ehrlich Ascites Carcinoma, Antineoplastic activity, cell growth inhibition.

1. INTRODUCTION

Actinobacteria have been looked upon as potential sources of bioactive compounds and the works done earlier have shown that these microbes are the richest sources of secondary metabolites [1]. They possess a dominating position as targets in exploring study due to their diversity and ability to produce new, safe and effective molecules of pharmaceutical interest [2]. Since the discovery of actinomycin, actinobacteria have been found to produce many commercially bioactive compounds and natural pigments, in addition to enzymes of industrial importance [3]. The search for an effective anticancer drug always attracts the researchers to investigate the possibility of natural products in the treatment of cancer [4]. In this respect, Actinobacteria are economically and biotechnologically most valuable prokaryotes. They are considered for the production of about one half of the discovered compounds such as antibiotics, anticancer compounds and enzymes [5]. A large number of antitumor compounds produced by Actinomycetes are anthracyclines (aclarubicin, daunomycin and doxorubicin), peptides (bleomycin and actinomycin D), aerobic acids (mithramycin), enediynes (neocarzinostatin), antimetabolites (pentostatin), carzinophilin, mitomycins and others [6, 7]. The search for a biologically potent drug is still a superior goal for cancer treatment. The highly toxic action and undesirable side effects associated with chemotherapeutics demand for safe and greater therapeutic efficiency antitumor drugs [8]. However, the frequency of rediscovery of known compounds by *Streptomyces* strains was fairly high [9].

Thus, minimization of problems of the previously discovered compounds, cultivation of two different microbial strains together in one culture vessel i.e. competing co-culture allows direct interaction between these microbes which may lead to the induction of new secondary metabolites not previously observed in the independent culture of the strains [10]. This phenomenon might be due to microbial crosstalk or chemical defense mechanism leading to the switching on of cryptic biosynthetic genes in a second

microorganism as the result of a signal from the other microorganism [11,12]. From this standpoint, we co-cultured two *Streptomyces* spp.(one marine and another terrestrial) and studied the antineoplastic effect of co-cultured bacterial metabolites where % cell growth inhibition, mean survival time and hematological parameters was observed.

2. MATERIAL AND METHODS

2.1 Inoculums preparation and fermentation

A loop full of the organisms of ANTS-1 and AIAH-10 was transferred from preserved slant culture to a 100ml flask containing 60ml yeast extract glucose broth medium. The flasks were shaken in a rotary shaker at 220 rpm at 31^0C for 2 days. The inoculum of ANTS-1 & AIAH-10 was used as the seed culture. 20ml two days inoculums of AIAH-10 were mixed in a 500ml conical flask containing 200ml yeast extract glucose broth medium in the same process.

2.2 Recovery of the product from the culture medium

The co-culture of ANTS-1 & AIAH-10 in yeast extract glucose broth media was extracted by ethyl acetate. At first, the cell biomass was separated by filtration and then, the total filtrate was collected by solvent extraction. The ethyl acetate fraction thus obtained was evaporated under reduce pressure in a rotary vacuum evaporator at 45^0C until a solid reddish-brown mass was obtained. It was transferred to a previously weighed small beaker using ethyl acetate. After evaporation of the solvent EA, the weight of the solid extract was determined. This was called 'crude extract' of co-cultured metabolites namely 'AIKA-110'.

2.3 Cell growth inhibition study

The effect of 'AIKA-110' against Ehrlich Ascites Carcinoma (EAC) cells in Swiss albino mice (female) was investigated. Swiss albino mice of 5-7 weeks old, weighing 20-26 grams collected from ICDDR'B, Mohakhali, Dhaka. Transplantable tumor (EAC) was used in this experiment. The initial inoculums of EAC cells were provided by the Indian Institute of Chemical Biology (IICB), located at Kolkata, India. The EAC cells were propagated in laboratory biweekly intraperitoneal (i.p.) injections of 3×10^6 cells, freshly drawn from a donor Swiss albino mice bearing 6-7 days old ascites tumor suspended in 0.3ml sterile saline solution. Therefore, for the transplantation of Ascites tumor to mice the freshly drawn fluid was diluted with normal saline (0.98%) NaCl solution and the tumor cells number was adjusted to approximately 2×10^6 cells/ml by counting cell number with the help of a hemocytometer using the following formula.

No. of cells per ml = The average count per square × Dilution factor / Depth of fluid under cover slip × Area counted.

The viability of tumor cells was observed by trypan blue dye (0.4%) exclusion assay. Cell sample showing above 90% viability was used for transplantation [13]. Tumor suspension of 0.1ml was injected intraperitoneally to each mouse. The strict aseptic condition was maintained throughout the transplantation process. For treatment, the stock solution of bleomycin was made by using distilled water at the concentration of 0.075mg/ml. AIKA-110 was dissolved in pure distilled water at the concentration of 50mg/ml and 100mg/ml. Four groups of mice (6 in each group) were used. For therapeutic EAC cells in every in every mouse were inoculated into each group of mice on day '0'. Treatments were started after 24 hours of tumor inoculation & continued for five days. Group one to two received the test compound of the doses of 50mg/kg (i.p.) and 100mg/kg (i.p). Group three received bleomycin at the dose 03.0mg/kg (i.p.), and group four was used as a control. Mice in each group sacrificed on day six, and the total intraperitoneal tumor cells were harvested by normal saline. Viable cells were first identified by using trypan blue and then counted by a hemocytometer.

2.4 Determination of percentage (%) increase of life span

Mean survival time of Swiss albino mice was determined by the method described by Sur et al., 1994. Four groups of mice (6 in each) were used. For therapeutic evaluation 136^6 EAC cells per mouse were inoculated into each group of mice on day '0'. Treatment was started after 24 hours of tumor cell inoculation and continued for 10days. Tumor growth was monitored, and host survival was recorded and expressed as mean survival time in days and % increase in life span was calculated by using the following formula:

MST= ∑ Survival time of each mouse in a group/ Total no. of mice

% Increase of life span = (MST of treated group/ MST of control group) −1 × 100

2.5 Effect of AIKA-110 on hematological parameters

In the case of tumor-bearing mice, treatment was started after 24 hours of EAC cell transplantation and continued for ten days. Blood was drawn from each group of mice on day 6 by the tail puncture. Hemoglobin % was measured by using Sahlins hemocytometer. Total WBC and RBC cells were counted.

3. RESULTS

Treatment with bacterial metabolites resulted in maximum cell growth inhibition at the doses 100mg/kg (i.p.) and 50mg/kg (i.p.) as evident from 74.66% and 47.67% reduction of tumor cells respectively (Figs 1 & 2). Treatment at the dose of 0.3 mg/kg with bleomycin showed cell growth inhibition by 89.64% (Table 1)

Fig. 1. EAC cell of control mice

Fig. 2. EAC cell of mice treated with 50 mg/kg

Fig. 3. EAC cell of mice treated with 100 mg/kg

Table 1. Effect of AIKA-110 on EAC cell growth inhibition (in vivo)

Name of Exp.	Nature of the Drug	Dose in mg/kg/day (IP)	No of EAC cells in mouse on day six after tumor cell inoculation	% of cell growth inhibition
Control	–	–	$(1.93 \pm 0.12) \times 10^{7}$	–
Bleomycin	Standard	0.3 mg/kg	$(0.20 \pm 0.03) \times 10^{7**}$	89.64
AIKA-110		50 mg/kg	$(1.01 \pm 0.18) \times 10^{7*}$	47.67
		100 mg/kg	$(0.489 \pm 0.05) \times 10^{7**}$	74.66

Number of mice in each case (n=6); the results were shown as mean **± SEM. Where significant values are *p<0.05 and **p<0.01.**

It has been observed that tumor-induced mice treated with AIKA-110 at doses 50mg/kg (i.p.) and 100mg/kg (i.p) resulted in increase of life span 43.59% & 70.76% respectively, when compared to that of control mice. Bleomycin increased life span by 82% when compared with control mice (Table 2 and Fig 3)

Table 2. Effect of bacterial metabolites on survival time of EAC cells bearing mice.

Name of Exp.	Nature of the Drug	Dose in mg/kg/day (IP)	Mean survival time (Days) Mean ± SEM	% Increase of life span % ILS
Control	**–**	**–**	**19.5 ± 1.20**	**–**
Bleomycin	**Standard**	**0.3 mg/kg**	**35.5 ± 0.32**	**82.00*** **
AIKA-110		**50 mg/kg**	**28.0 ± 0.61**	**43.59***
		100 mg/kg	**33.3 ± 0.52**	**70.76****

Number of mice in each case (n=6); the results were shown as mean **± SEM. Where significant values are *p<0.05 and **p<0.01.**

Hematological parameters were found to be altered from normal values along with the growth of the tumor. Hemoglobin, RBC & WBC count were found to be changed after inoculation of EAC cells in Swiss albino mice. After treatment with 'AIKA-110' at doses 50 & 100, it is found that the parameters restored moderately only at high doses (Table 3)

Table 3. Effect of bacterial metabolite on hematological parameters in tumor-bearing mice on day 14 of tumor inoculation

Name of Exp.	RBC (cells/ml)	WBC (cells/ml)	Hb (gm/dl)
Normal mice	$(3.12\pm0.2)\times10^{9}$	$(10.1\pm1.4)\times10^{6}$	22.2±.04
Control (EAC bearing mice)	$(1.93\pm.4)\times10^{9}$	$(24.0\pm0.3)\times10^{6}$	19±.37
EAC+50 mg/kg Bacterial Metabolite	$2.07\pm.22\times10^{6}$	$(17.5\pm0.63)\times10^{6}$	19.1±13
EAC+100 mg/kg Bacterial Metabolite	$2.48\pm.14\times10^{6}$	$(14.1\pm0.13)\times10^{6}$	20.2±03

4. DISCUSSION

Treatment of cancer depends on surgery, chemotherapy, and radiotherapy. By investigation of various drugs or surgical methods the prognosis of cancers can be improved [14,15,16].The antineoplastic activity of AIKA-110 was judged by measuring cell growth inhibition and enhancement of life span of the EAC cell-bearing mice. The efficiency of AIKA-110 was compared with the standard anticancer drug, bleomycin at the dose of 0.3mg/kg (i.p.). AIKA-110 inhibits the cell growth rate effectively, 74.66% inhibition was achieved at dose 100mg/kg (i.p) which is quite comparable to that of bleomycin (0.3mg/kg). AIKA-110 also increased the life span of tumor-bearing mice effectively. The potency was found to be increased with the enhancement of dose. In the present study, a 70.76% increase in the life span of tumor-bearing mice at dose 100mg/kg (i.p.) was observed with AIKA-110. Further enhancement of the life span is therefore expected with still higher doses and it is considered as an important parameter for judging the suitability of a compound as an anticancer agent [17,18]. AIKA-110 increases the total RBC & decreases WBC & increases hemoglobin concentration that is quite comparable with the standard bleomycin drug at the dose of 0.3mg/kg.

5. CONCLUSION

Therefore, it may be concluded that AIKA-110 contains a potent antineoplastic compound(s) which would be an important bioactive constituent(s) for combating fatal diseases after isolation and identification

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