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# *IN VIVO* EVALUATION OF ANTICANCER ACTIVITY OF NOVEL 6-FLUORO-3-(PIPERIDIN-4-YL) BENZO [D] ISOXAZOLE DERIVATIVES AGAINST EHRLICH ASCITES CARCINOMA IN MICE

**Sharath Chandra SP[1]* and Periyapatna Vishwaprakash Mahadimane[2]**
[1]Government Science College, Hassan, Karnataka, India
[2]Deparement of Bioscience, University of Mysore, Hassan, India

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**Abstract:** The aim of the present study is to investigate the anticancer properties of newly synthesized benzisoxazole derivatives (S1-S4) in Swiss albino mice against Ehrlich Ascites Carcinoma (EAC) cell line. The anticancer activity of synthesized molecules and 5 fluorouracil was investigated against Ehrlich ascites carcinoma (EAC) tumor in mice at dose of 20 mg/kg body weight (orally and intra peritoneally). Acute oral toxicity studies were performed to ensure the safety of the molecules. EAC cells were injected (i.p.) into ninety six mice (divided into 6 equal groups), and after a one-day incubation period, both molecules and drug was administered to the mice daily for 14 days. On day 15, 6 animals in each group were sacrificed for observation of anticancer activity like mean increase in body weight and viability of cancer cells. Whereas mean survival rate and percentage of increase in life span was conducted for a period of 6 weeks. Administration of the synthesized molecules resulted in a significant ($p < 0.05$) decrease in tumor volume and viable cell count, and increased non-viable cell count and mean survival time, resulting in increase of the life span of the EAC-bearing mice. The results indicate that the synthesized molecules (S2 and S3) exhibited significant anticancer activity comparable to that of standard drug 5 fluorouracil.

**Key words:** Anticancer, Benzisoxazole, EAC, Viable cells, Tumor volume

## INTRODUCTION

Cancer chemotherapy targeting tumor progression represents one of the most relevant challenges of chemists and oncologist since last few decades. In order to gain new insights into the complexity of the disease, robust screening methods for evaluating different natural or synthetic drugs have been carried out from the science community.

In this regard, heterocyclic compounds bearing piperidine skeleton are potential targets of organic synthesis owing to their pharmacological activity and their wide existence in nature[1]. Compounds with piperidine structures are known to possess antimicrobial[2], anti-inflammatory[3], local anesthetic[4] and their derivatives piperidines are also biologically important and act as neurobinin receptor antagonists[5], analgesic and antihypertensive agents[6]. Benzisoxazole derivatives containing piperidine moiety are one of the most important heterocyclic systems in the field of medicinal chemistry and associated with the wide range of biological activities, such as antimicrobial[7], anticancer[8,9], antipsychotics[10-12] properties. In this direction we have synthesized[13] 6-fluoro-3-(piperidin-4-yl)benzo[d]isoxazole derivatives, namely 4-(6-fluorobenzo [d] isoxazole-3-yl)-N-(3-methoxyphenyl) piperidine-1-carbothiamide (S1), N-(2-chlorophenyl)-4--(6-fluorobenzo [d] isoxazole-3-yl) piperidine-1-carbothiamide (S2), 4- (6-fluorobenzo[d]isoxazole-3-yl)-N-(2-fluorophenyl)) piperidine-1-carbothiamide (S3), N-(4-chlorophenyl)-4-(6-fluorobenzo[d] isoxazole-3-yl) piperidine-1-carbothiamide (S4). Thus numerous pharmacological activities of Benzisoxazole derivatives prompted us to evaluate the anti-cancerous activity of newly synthesized compounds using various in vivo assays.

***Corresponding Author:**
**Dr. Sharath Chandra SP,**
Assistant Professor and Head,
Department of Biochemistry,
Government Science College,
Hassan, Karnataka, India.

## MATERIALS AND METHODS

### *Animals*

Line bred female Swiss albino mice, 6-8 weeks old, weighing 20-25g were used for the present study. They were housed in standard environmental condition like, ambient temperature (25°C ± 1°C), relative humidity (55±5%), and 24hr light dark cycle. Animals had free access to standard pellet diet and water ad libitum. All animal experiments were carried out in accordance with the guidelines of CPCSEA. The institute animal ethical committee has given the approval for the animals and the studies on them (approval No. HSK CP/IAEC/12013-14/121).

### *Acute Toxicity Study*

The dose selection was done out according to safe dose calculation as indicated in acute toxicity studies (OECD TG420) for the drug and samples S1 to S4. Acute toxicity studies gave similar results like that of anticancer drug. With this basis, we selected the dose 20 mg/kg for further studies.

### *Tumor cells*

Ehrlich Ascites Carcinoma (EAC) cells are mouse mammary carcinoma cells that grow as ascites tumor in peritoneal cavity of mice. EAC cells were grown in the peritoneal cavity of six to eight weeks old Swiss albino mice by peritoneal transplantation of

0.5ml of cell suspension contain 1× $10^6$ cells in sterile saline (0.9%NaCl).

***Experimental design***

Male Swiss albino mice were divided in to 6 groups containing 12 animals in each group. The entire groups were injected with EAC cells (1X$10^6$cells/mouse) intraperitoneally.

**Group I:** Induced EAC cell (1 X$10^6$) with DMSO (0.9%)
**Group II:** Induced EAC cell (1X$10^6$) with 5fluorouracil (20mg/kg i.p)
**Group III:** Induced EAC cell (1X$10^6$) with S1 (20mg/kg p.o) with DMSO (0.9%)
**Group IV:** Induced EAC cell (1X$10^6$) with S2 (20mg/kg p.o) with DMSO (0.9%)
**Group V:** Induced EAC cell (1X$10^6$) with S3 (20mg/kg p.o) with DMSO (0.9%)
**Group VI:** Induced EAC cell (1X$10^6$) with S4 (20mg/kg p.o) with DMSO (0.9%)

***Mean survival time and Percentage increase life span***

The effect of synthetic compound (S1-S4) on tumor growth was monitored by recording the mortality daily for a period of 6 weeks and percentage increase in average life span was calculated.
Mean survival time (MST) = (day of 1$^{st}$ + day of last death)/2

% ILS = {(life span of treated group/ life span of controlled group)-1} X 100

***Body weight analysis***

Body weights of the experimental mice were recorded both in the treated and control groups at the beginning of the experiment (day 0) and sequentially on every 5th day during the treatment period and calculated on 15th day.

***Determination of tumor volume and packed cell volume***

The mice were dissected and the ascetic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and weight immediately. The ascetic fluid was collected from the peritoneal cavity. The packed cell volume was measured by taking it in a graduated centrifuge tube and by centrifuging at 1000 rpm for 5 min.

***Tumor viable cell count***

The ascetic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber. The cells were then stained with tryphan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable and those that took the stain were nonviable. These viable cells were counted in the 64 small squares.

***Statistical analysis***

All values were expressed as mean ± SEM. Statistical analysis was performed with one way analysis of variance (ANOVA) followed by DMRT. P value < 0.05 was considered as significant when compared to control.

## RESULTS

In all the cases, mean values of the repeated experiments were taken and represented as mean± SE. The acute toxicity of synthetic compounds was found to be 240mg/kg for the p.o treatment of male albino mice. The antitumor activity of synthetic compounds (S1-S4) against EAC tumor bearing mice was assessed by the parameters such as survival time, %ILS, tumor volume, packed cell volume, increase in body weight and tumor cell count (viable and nonviable cell).

***Mean survival time and Percentage increase life span***

In the EAC control group, the mean survival time (Table 1) was 20.17 ±0.70 days, while it increased to 22.17 ±0.40 (S1), 30.83 ±0.48 (S2), 27.67 ±0.42(S3) and 24.67 ±0.33 (S4) days, respectively in synthetic compounds treated groups, whereas the standard drug 5-fluorouracil (20mg /kg)-treated group had a mean survival time of 38.50 ±0.89 days. Similarly the percentage of increase in lifespan was observed highest in S2 (52.89%) and S3 (37.19%) in groups treated with synthesized molecules, when compared to the standard drug (92.56%) treated groups.

**Table 1:** Effect of Synthetic compounds (S1-S4) and standard drug on Mean survival time and increase in life span in EAC bearing mice

| Parameter | Control EAC Control | Standard 5-fluorouracil | S1 | S2 | S3 | S4 |
|---|---|---|---|---|---|---|
| Mean survival time(days) | 20.17 ±0.70 | 38.50 ±0.89 | 22.17 ±0.40 | 30.83 ±0.48 | 27.67 ±0.42 | 24.67 ±0.33 |
| Increase in lifespan (%) | - | 92.56 | 9.92 | 52.89 | 37.19 | 22.31 |

***Body weight analysis***

Figure 1 indicates that a remarkable decrease in body weight was observed in standard drug 5-fluorouracil (4.21 ±0.09), S2 (7.07 ±0.19) and S3 (7.97 ±0.18) when compared to the control groups (12.33 ±0.49). However S1 (11.02 ±0.17) and S4 (9.63 ±0.22) also showed lesser values than the control groups.

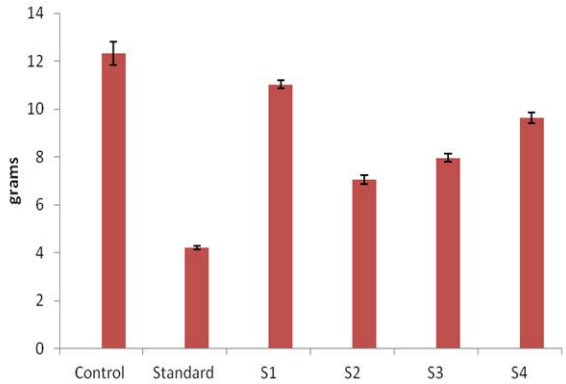


**Figure 1:** Effect of Synthetic compounds (S1-S4) and standard drug on Body weight

***Determination of tumor volume and packed cell volume***

In the Figure 2 values of standard drug (2.67 ±0.42), S2 (5.92 ±0.05) and S3 (6.22 ±0.10) showed significant decrease in tumor volume when compared to the control groups (10.95 ±0.31). The values of S1 (8.23 ±0.14) and S4 (7.68 ±0.09) also indicate a decrease in tumor volumes. The packed cell volumes of S2 (2.28 ±0.07) showed similar results as that of standard drug 5-fluorouracil (2.17 ±0.31). However the findings of other synthetic molecules S3 (2.58 ±0.07), S1 (3.43 ±0.08) and S4 (3.27 ±0.06) have shown better results when compared to the control groups (5.83 ±0.60).

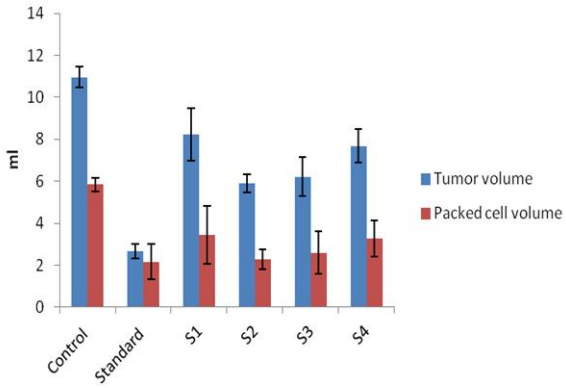


**Figure 2:** Effect of Synthetic compounds (S1-S4) and standard drug on Tumor volume and packed cell volume

***Tumor viable cell count***

The viable cell count as seen in the Figure 3 indicates significant results for the standard drug 5-fluorouracil (22.33 ±0.33), however there was also visible difference in the decrease in the viable cell of count S2 (53.79 ±0.43) and S3 (56.52 ±0.92) when compared to the control groups (95.67 ±0.49). Non-viable cell count also increased drastically in anticancer drug (93.00 ±0.86), S2 (50.83 ±0.48) and S3 (47.83 ±1.01) when compared to the control groups (12.17 ±0.31). Better values were also seen in S3 and S4 for both viable and nonviable cell count values when compared to the control groups.

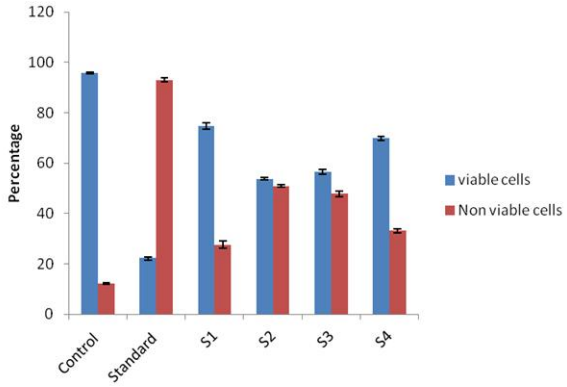


**Figure 3:** Effect of Synthetic compounds (S1-S4) and standard drug on percentage of viable and non-viable EAC cells

## DISCUSSION

The present investigation was undertaken to evaluate the anticancer properties of 6-fluoro-3-(piperidin-4-yl) benzo [d] isoxazole derivatives (S1-S4), in EAC bearing mice. EAC cells are one of the rapidly growing tumor cells and are able to grow in almost all strains of mice[14]. Ascetic tumor implantation promotes local inflammatory reactions leading to increase in vascular permeability and resulting in intense edema formation, cellular migrations and progressive ascetic fluid formation. Biological chemicals, such as interferons and interleukins present a nonspecific immunity against indirect cytotoxic mechanisms. Anticancer properties[15] of interferons and interleukins are complex which includes promotion of differentiation, immunomodulation, anti-proliferative property, inhibition of angiogenesis and activation of oncogene. Interleukins are responsible for the growth and activity of immune cells, which act as anticancer agents by affecting the cytolytic activity of T-lymphocytes and natural killer cells and activating the gene regulation responsible for cancer cell death.

In Ehrlich acites carcinoma cells bearing mice, an increase in ascetic tumor volume was noted, as acites fluid is the direct nutritional source for tumor cells. A rapid increase in ascetic fluid with tumor growth would be a means to meet nutritional requirement of tumor cells[16]. The potency of synthetic derivatives (S1-S4) as anticancer agents along with standard drug 5-flurouracil has been judged by

measuring many parameters. The increase in the mean survival time[17] of S2 and S3 comparable to that of standard drug indicates the potential of the synthesized molecules to protect the cells from early cell death. The findings indicated significant increase in life span of EAC-bearing mice upon treatment with standard drug and synthesized molecules S2 and S3. The extension of life span of EAC bearing mice is a crucial criterion[18] for considering the promising role of any molecule as anticancer agent. There was also significant decrease in tumor volume (mL) in S2 and S3 treated groups when compared to the control groups. The packed cell volume values of synthesized molecules S2 and S3 also showed similar results to that of the groups treated with standard drug. This may suggest the delay in vascular permeability to the cells[19], thus indicating the role of synthesized molecules (S1-S4) in tumor biology. Decrease in viable cell count and higher non-viable cell count noticed in groups treated with synthesized molecules, particularly S2 and S3 and groups treated with drugs suggests that the above molecules stimulate the immune cells by the producing Interleukins, which target cancer cells and lead to cancer cell death by indirect cytotoxic mechanism. The standard drug 5 fluorouracil when transformed into several cytotoxic metabolites gets incorporated into DNA leading to cell cycle arrest and apoptosis. The above mechanism disturbs the cells ability to generate DNA. Thus the above results may indicate that the synthesized molecules showing comparable results to that of standard drug may follow the same mechanism in preventing cancer cell growth.

## CONCLUSION

Based on the above findings, it can be concluded that the synthesized molecules (S1-S4), S2 and S3 in particular showed comparable anticancer activity as that of standard drug 5 fluorouracil against EAC cells in Swiss albino mice. However, the in vivo evaluation of synthesized molecules is insufficient to use them as novel anticancer medications. Furthermore anticancer studies have to be carried out using different cancer cell lines and higher animal models.

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