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SHORT COMMUNICATION

A combination of semi-purified L-methioninase with tamoxifen citrate to ameliorate breast cancer in athymic nude mice

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Abstract

Background Chemotherapy nonspecifically targets both tumor and healthy proliferating cells. Methionine deprivation using L-methioninase along with chemotherapy appears promising towards cancer management. The present study is an attempt to use a new combination of L-methioninase with Tamoxifen (TAM) to treat breast cancer in mice.

Methods and results L-Methioninase from *Methylobacterium* sp. was partially purified (SPMet's) by cold acetone precipitation and lyophilized. Its cytotoxicity effect, alone and in combination with Tamoxifen, was evaluated *in vitro* (MCF-7) cells and *in vivo* (athymic nude mice) conditions. SPMet's was found to inhibit the growth of MCF-7 cells with an IC_{50} value of 47.05 µg/ml, while the combination of SPMet's and TAM had an IC_{50} of 6.4 µg/ml. Athymic nude mice were grouped into: Group-I - Tumor control; Group-II - TAM; Group-III - SPMet's; Group-IV - SPMet's + TAM. Tumor growth inhibition (TGI) was maximum in Group-IV with 84.65% followed by Group-II with 65.12%. Hematological and Biochemical parameters in Group-II, III, and IV were restored to normal levels. Tumor histopathology showed increased apoptosis and necrosis in Group-IV. Caspases 3 & 8 gene upregulation was significantly higher in Group-IV than other treated groups, indicating higher efficacy of the combination approach.

Conclusion This is the first study report about a combination of SPMet's and TAM on *in vivo* breast cancer model, with significantly higher anticancer activity and without noticeable side effects. The findings of this study have several important implications for future clinical studies.

Keywords Combination approach · Chemotherapy · L-Methioninase · SPMet's · Tamoxifen · Breast cancer · Athymic nude mice

Abbreviations

SPMet's	Semi purified L-methioninase
TAM	Tamoxifen citrate
MCF-7	Breast cancer cell line
HEK-293	Human embryonic kidney cell line
TGI	Tumor growth inhibition
PVC	Packed cell volume
MCV	Mean corpuscular volume
MCH	Mean corpuscular hemoglobin
M.C.H.C	Mean corpuscular hemoglobin concentration
SGOT	Serum glutamate oxaloacetic transaminase
SGPT	Serum glutamate pyruvic transaminase
ALP	Alkaline phosphatase
BUN	Blood urea nitrogen
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
rMET	Recombinant methioninase

Introduction

Large number of cancer related deaths are linked to breast cancer. The treatment for breast cancer is usually through multiple ways: surgical, medical, and radiological interventions or through combination of more than one of the listed ways, to minimize the death rate and enhance the effectiveness of the treatment [1]. Though chemotherapy is the preferred choice, drug resistance posed by cancer cells is

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a challenge, besides the series of side effects like weight loss, variation in biological parameters, lethargy, anemia, and others. Hence, developing alternative therapeutic approaches is always a priority research.

Extensive research since 1973 has established the fact that cancer cells are methionine dependent, as they exhibit high levels of transmethylation leading to increased Methionine uptake. Down regulation of o-6-methylguanine-DNA methyltransferase and reduced S-adenosylmethionine (SAM) by methionine deprivation decreases DNA methylation, alters DNA stability, and thus causes increased sensitivity of cancer cells towards DNA damaging agents [2]. This relation between Methionine and Methylation in cancer is referred to as the Hoffman effect. Excessive utilization of methionine was observed in [^{11} C] methionine PET imaging of tumors when compared to normal tissues. It has been reported that methionine dependence is more specific to tumors as compared to glucose dependence [3, 4]. Targeting cancer cells by methionine deprivation leads to inhibition of cancer growth by arresting the cells in the S/G2 phase, that eventually undergo apoptosis [5]. As per some reports, drug resistance is mainly due to cancer stem cells, but even these cells are susceptible to methionine deprivation [6]. This methionine deprivation strategy using Methioninase enzyme was reported to sensitize the tumor cells to chemotherapeutic drugs as well [7]. Using a combination approach of L-Methioninase along with a chemotherapeutic drug is appearing as more promising for cancer therapy than monotherapy [8, 9]. Recently a clinical trial indicated that oral rMETase and a low-methionine diet together is effective in rectal cancer [10]. Tamoxifen (TAM) has shown benefit for all groups of breast cancer patients, hormone sensitive tumors, and even reported for its beneficial effects in the prevention of breast cancers [11]. However, no studies have been attempted using L-Methioninase in combination with TAM for breast cancer.

In the present study, we analyzed a new combination of TAM along with the semi-purified Methioninase (SPMet's) enzyme isolated from a novel *Methylobacterium* sp. on both *in vitro* breast cancer cell line and *in vivo* breast cancer mice model.

Materials and methods

Cell culture and maintenance

Breast cancer cell line MCF-7 was procured from ATCC, USA. They were cultured and maintained in DMEM media supplemented with 10% Fetal Bovine Serum (HIMEDIA, India), penicillin (100IU/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO2 at 37 °C.

Cytotoxicity and toxicity effect of SPMet's.

L-Methioninase enzyme was isolated and purified from a bacterium, *Methylobacterium* sp. JUBTK33 [12]. The cytotoxic effect of SPMet's was studied on MCF-7 and HEK-293 cell lines, following the standard MTT assay [13]. SPMet's at different concentrations- 25, 50, 100, 200 and 400 µg/ml, alone and in combination with TAM (5 and 10 µg/ml) were used, while Doxorubicin (5 µg/ml) was used as the positive control.

In vivo analysis of MCF-7 cell line induced breast cancer on athymic nude mice

Mice maintenance

Athymic nude mice were procured from Charles River, USA and housed in individually ventilated cages with three mice per cage. Breast cancer was induced by injecting MCF-7 cells subcutaneously, at the region above the right flank. Throughout the study, mice were maintained at 22ºC (+ 3ºC) temperature and relative humidity of 50–60% with artificially induced lighting of 12 h light, and 12 h dark. Mice received Methionine-free diet procured from PMI nutrition international, USA. Every day, freshly prepared pyridoxal phosphate 100 µmol/L was mixed with fresh drinking water and given to mice. Feed and water were given *ad libitum.* The study was Approved by the Institutional Animal Ethics Committee (IAEC) with IAEC approval no IAEC-SLS-2020-010. All experiments were carried out in a BSL-2 biosafety cabinet, all the reagents and consumables used were pre-sterilized.

Experimental design and tumor inhibition analysis

Breast cancer model mice were divided into Group-I: Control (normal saline); Group-II: TAM 20 mg/kg; Group-III: SPMet's (100 mg/kg); Group-IV: SPMet's (100 mg/kg) with TAM (20 mg/kg). The drug was administered orally throughout the study for 14 days.

Hematological and bochemical parameters

At the end of the study, mice were sacrificed with Ketamine and Xylazine at 45 mg/kg and 4.5 mg/kg b.wt. Approximately 0.6ml – 0.7ml of the whole blood collected retro-orbitally was subjected to CBC analysis using a semi-automated Hematology analyzer (Erba transgesia; H360). The serum separated was used for biochemical analysis using a semi-automated biochemistry analyzer (Erba, Chem5X).

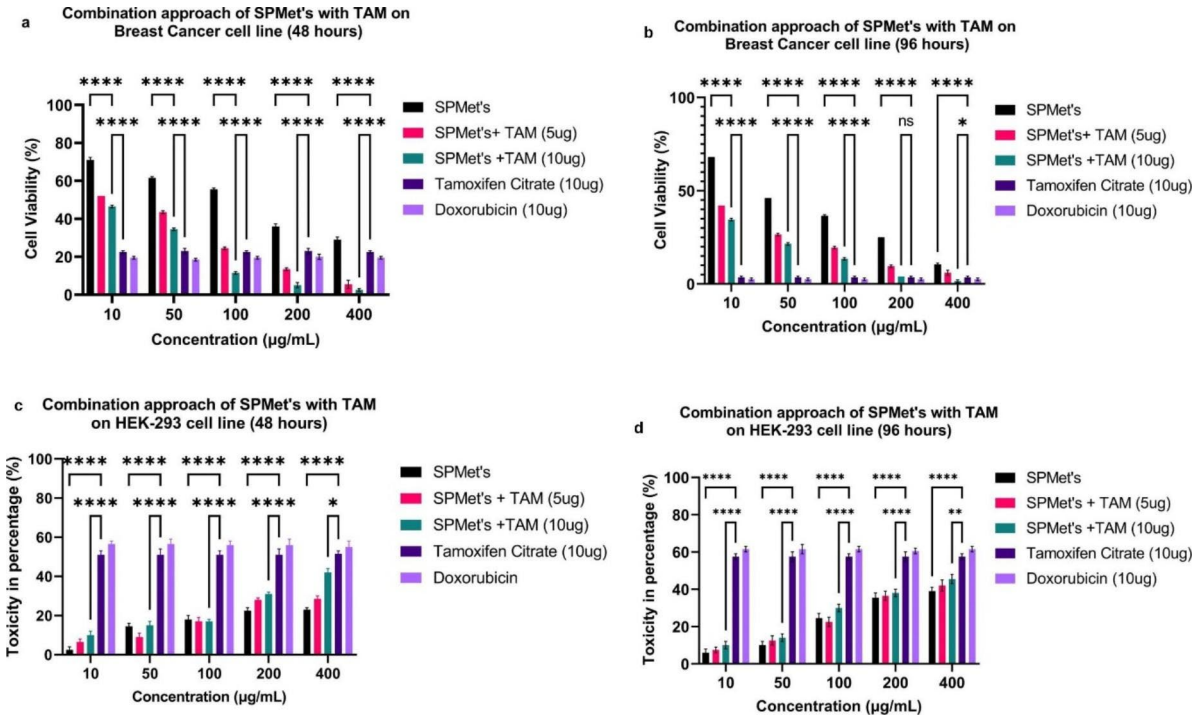

Fig. 1 The effect of SPMet's in combination with TAM: **(a)** Cytotoxicity on MCF-7 cell line at 48 h; **(b)** Cytotoxicity on MCF-7 cell line at 96 h; **(c)** cytotoxicity on HEK-293 at 48 h; **(d)** cytotoxicity on HEK-293 at 96 h. Values are represented as mean ± SD. ****p < 0.0001; **p < 0.01; *p < 0.05; ns- p > 0.05

Quantitative gene expression study by real-time PCR for caspase 3 and caspase 8

The tissue was processed using a standard kit-based assay protocol for RNA isolation, cDNA was synthesized using the Prime Script RT reagent kit (TAKARA) and caspase 3 and 8 gene expressions were analyzed as per standard methods [14].

Analysis of apoptosis through FITC-annexin V staining and FACS.

Identification and quantification of apoptotic cells by flow cytometry was performed by conjugating FITC to Annexin V and staining the cells with FITC-Annexin V and propidium iodide [15].

Histopathology studies

The tissue sections of Tumor, Liver, Kidney, Lungs, and Heart were subjected to Histopathology examinations using Standard methods. Photo-imaging was performed using Labomed binocular microscope Model Lx300 [16].

Statistical analysis

Statistical significance of each test was performed in the Graph Pad prism software to determine the significance of the study with p-value being < 0.05. All experiments were carried out in triplicate samples and analyzed statistically using analysis of variance. The data were represented as mean ± SD.

Results and discussion

In vitro analysis of cytotoxicity and toxicity of SPMet's

Cytotoxicity results showed that SPMet's (400 µg/ml) in combination with TAM showed higher cytotoxicity at 96 h. We observed SPMet's, TAM and DOX treatments resulting in 18%, 4% and 3% cell viability respectively in monotherapy (Fig. 1a). Whereas, in the combination approach of 400 µg/ml SPMet's + 10 µg/ml TAM, we found significant inhibition of MCF-7 cells with 1% viability (p value- 0.008). On healthy HEK-293 cells, we observed viabilities of 39% with DOX, 57% with TAM 10 µg/ml, 44% in SPMet's + TAM 10 µg/ml, 41% in SPMet's + TAM 5 µg/ml, and 61% in SPMet's (Fig. 1b). We found the combination to be significantly reducing the toxicity of TAM by 13% (p-0.0004),

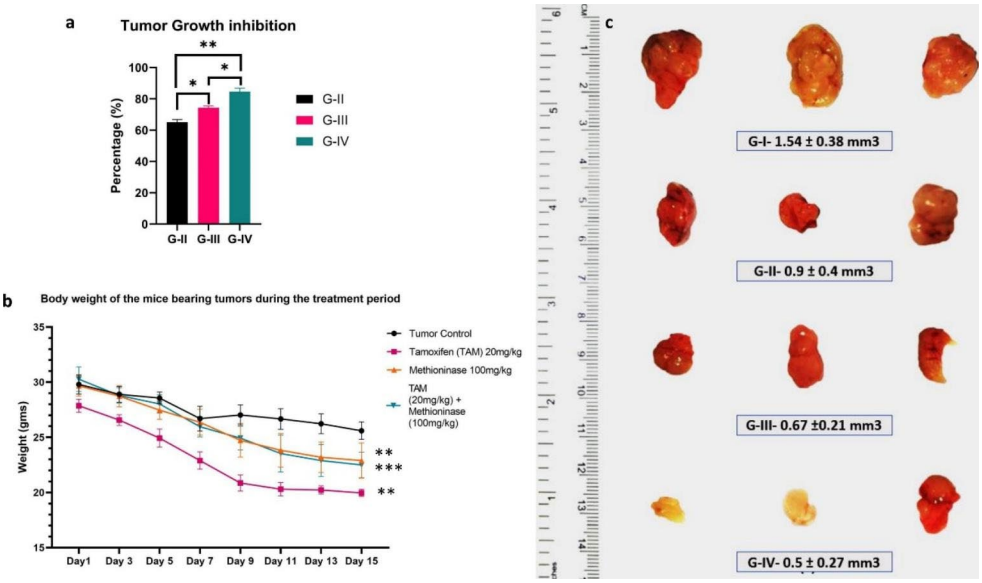

Fig. 2 Tumor evaluation **(a)** Evaluation of body weight of tumor bearing mice; **(b)** Tumor growth inhibition; **(c)** Photographs of Breast cancer tumor at the end of the treatment. Values are represented as mean ± SD. ***p < 0.001; **p < 0.01; *p < 0.05

thus indicating the combination approach as highly efficient in minimizing the side effects of monotherapy.

In vivo study of MCF-7 induced breast cancer on athymic nude mice

Tumor growth inhibition

The Athymic nude mice were randomized into 4 different groups with n = 3. On 15th day after treatment, we observed tumor volume inhibition with TAM- 690.42 ± 245.15 mm^3, SPMet's-509.46 ± 128.66 mm^3, SPMet's + TAM-303.9 ± 167.37 mm^3 in treatment groups, as compared to tumor control (Group I) with a tumor volume of 1979 ± 299.05 mm^3 (Fig. 2a, 2c). The combination therapy was found to be significantly effective in tumor growth inhibition (TGI) with 84.65% (p-0.0001) than monotherapy of TAM with 65.12% (p-0.002), SPMet's with 74.26% (p-0.001). This TGI is much higher than that reported by Hoffman et al. with 52.74% reduction in tumor volume using oral rMETase [4]. Significant tumor volume inhibition was found with slight weight loss in the mice of all treatment groups when compared with the tumor control mice (Fig. 2b). The effective anticancer activity of combination approach might be due to methionine restriction which enhances selective arrest of the cancer cells in S/G2 phase of cell cycle making them susceptible for chemotherapeutic drugs [17]. Body weight increase was more in control mice than the treatment groups, which did not exhibit major increase in their bodyweights. Similar observation was reported by using o-rMETase + 5-FU + OXA and methioninase from *Trichoderma hazarium* [18, 19].

Hematological and biochemical analysis

The major issue with chemotherapy is anemia and myelosuppression. Since anemia is a major concern during cancer treatment [20], reduction in RBC and Hemoglobin are commonly observed among cancer patients. In the current study, we observed significant improvement in the levels of Hb with 12.90 gm% and RBC with 22.23 (mill/cumm) in combination approach when compared with tumor control with Hb 12 gm% and RBC-12.10 (mill/cumm) (Table 1). All the other parameters were found to be maintained in the normal range throughout the study. These results indicate that combination therapy has a protective effect on the Hematopoietic system and was effective in improving the hematological conditions.

The presence of tumor either in mice model or humans, causes loss of functional integrity in the Liver [21]. Contrary to this, in our study we found significantly elevated levels of liver enzymes Bilirubin, SGOT/AST, SGPT/ALT, and ALP in untreated group (Table 2). These elevated levels generally indicate the damage caused to the liver due to tumor burden. Through histopathology analysis, it was found that all organs were healthy in all the treatment groups, but in the untreated tumor control, the liver was affected (Fig. 3a-d). The presence of intracytoplasmic vacuoles in the hepatocytes were observed, and the liver weighed 1.54 ± 0.42 gm in Group-I (Fig. 3e). On the contrary, in the case of all treatment groups we found liver weighed approximately 1.0 gm, indicating the promising hepatoprotective nature of SPMet's. The combination approach was successful in restoring the biochemical and hematological parameters to the normal range. Such effects were reported earlier too, where they studied monotherapy of L-Methioninase isolated from *Trichoderma*

Table 1 Evaluation of Hematological parameters of all the treatment groups

Parameters	Group-I **Tumor control**	Group-II **Tamoxifen 20 mg/kg**	Group-III **SPMet's 100 mg/kg**	Group-IV **TAM 20 mg/kg + SPMet's**
Hb (gm%)	12 ± 2.19	12.83 ± 0.31	12.73 ± 0.15	12.9 ± 0.89
WBC count cells 10^9/L)	2.36 ± 0.5	5.30 ± 1.51	6.16 ± 2.3	6.0 ± 1.8
Neutrophils (%)	76.67 ± 5.77	71.67 ± 5.77	61.33 ± 12.06	75 ± 5.01
Lymphocytes (%)	12.33 ± 4.04	16.67 ± 2.89	32.67 ± 8.74	13.33 ± 2.89
Eosinophils (%)	7.77 ± 1.99	3.4 ± 0.49	4.97 ± 0.83	2.97 ± 0.2
Platelet count (Lakhs/cumm)	4.69 ± 1.4	7.49 ± 0.83	7.5 ± 0.48	7.97 ± 0.58
RBC count (mill/cumm)	12.1 ± 3.5	20.14 ± 1.35	16.9 ± 8.78	22.23 ± 8.2
PCV (%)	48.17 ± 5.13	46.03 ± 1.02	46.53 ± 1.25	46.63 ± 0.55
M.C.V (fl.)	17.33 ± 0.15	16.23 ± 0.64	16.1 ± 0.2	16.1 ± 0.5
M.C.H (pg)	36.43 ± 4.01	35.17 ± 0.7	34.73 ± 0.51	35.4 ± 0.26
M.C.H.C (%)	7.87 ± 2.19	12.83 ± 0.31	12.73 ± 0.15	12.9 ± 0.89

(All the data given are represented as Mean ± SD; n = 3; $p < 0.05$; Abbreviation: Hb: Hemoglobin, PVC: Packed cell volume, M.C.V: Mean corpuscular volume, M.C.H: Mean corpuscular Hemoglobin, M.C.H.C: Mean corpuscular Hemoglobin concentration)

harzianum and compared it with the chemotherapeutic drug 5-FU [16].

Analysis of apoptosis, gene expression and histopathology

As per flow cytometry analysis, maximum viability of tumor cells (82.52%) was in Group-I as compared to Group-II (39.89%) or Group III (60.87%) or Group IV (52.8%). Very few early and late apoptotic (4.9 & 3%) cells were found in Group I as compared to Group II (29.16% early and 18.17% late apoptotic cells) (Fig. 4a). As compared to group II and III, Group-IV exhibited 14.53% early apoptotic, 13.98% late apoptotic and 18.68% necrotic cells. We could observe significant reduction in tumor cell viability in combination approach when compared with Group-I ($p < 0.01$) with higher percentage cells in early apoptosis, late apoptosis, or necrosis. Through histopathological evaluation we observed combination approach to induce extensive tumor necrosis and apoptosis, whereas monotherapy with SPMet's showed higher necrosis and TAM showed higher apoptosis (Fig. 4c).

Altered apoptosis is a major indicator of cancer, where the key regulators for apoptotic responses are Caspase 3 and 8 [22]. In the present study, upregulation of Caspase 8 and Caspase 3 genes were observed in Group-II, III and IV as compared to Group-I (Fig. 4b). Caspase 8 upregulation was 1.34, 1.21, and 1.68-folds and Caspase 3 upregulation was 2.35, 2.19, and 3.28 folds in Groups II, III, and IV respectively. Higher expressions of Caspase 3 and 8 were seen in the combination approach, possibly be due to L-Methioninase mediated methionine depletion, which arrests the cancer cells in the G2 phase of the cell cycle that eventually undergo apoptosis instead of cell division [23–25].

The mice in all groups survived till the end of the study, with mice in Group-I and II demonstrating side effects like loss of appetite, weight loss and lethargy, but surprisingly, Group-III and IV mice were observed to be healthy, active and without any signs of weight loss, fatigue, or loss of appetite throughout the study. Similar observation was reported earlier with orally administered rMET on mice models regarding their body weights [26, 27]. In another

Table 2 Evaluation of Biochemical parameters of all the treatment groups

Parameters	Group-I **Tumor control**	Group-II **Tamoxifen 20 mg/kg**	Group-III **SPMet's 100 mg/kg**	Group-IV **Tamoxifen 20 mg/kg + SPMet's 100 mg/kg**
Total Bilirubin (mg/dl)	1.24 ± 0.27	0.42 ± 0.03	0.40 ± 0.07	0.36 ± 0.06
Total Protein (g/dl)	0.64 ± 0.48	0.21 ± 0.06	0.18 ± 0.04	0.20 ± 0.06
Serum Albumin (g/dl)	6.07 ± 0.32	5.43 ± 0.15	5.33 ± 0.23	5.30 ± 0.03
Serum Globulin (g/dl)	4.23 ± 0.61	3.13 ± 0.12	3.03 ± 0.55	3.00 ± 0.4
SGOT/AST (U/L)	100.00 ± 0.61	50.00 ± 11.79	44.00 ± 13.86	30.33 ± 8.39
SGPT/ALT (U/L)	79.67 ± 22.37	38.67 ± 5.51	35.33 ± 9.07	28.00 ± 8
ALP (/L)	125.33 ± 25.40	130.00 ± 18.03	140.00 ± 10	123.33 ± 19.5
Serum Creatinine (mg/dl)	2.03 ± 0.25	1.87 ± 0.4	1.83 ± 0.42	1.58 ± 0.3
BUN (mg/dl)	14.67 ± 3.51	13.37 ± 1.24	12.80 ± 0.98	12.73 ± 0.91

(All the data given are represented as mean ± SD; n = 3; $p < 0.05$; Abbreviation: SGOT: Serum Glutamic Oxaloacetic Transaminase, SGPT: Serum Glutamic Pyruvic Transaminase, ALP: *Alkaline phosphatase, BUN: Blood urea nitrogen)*

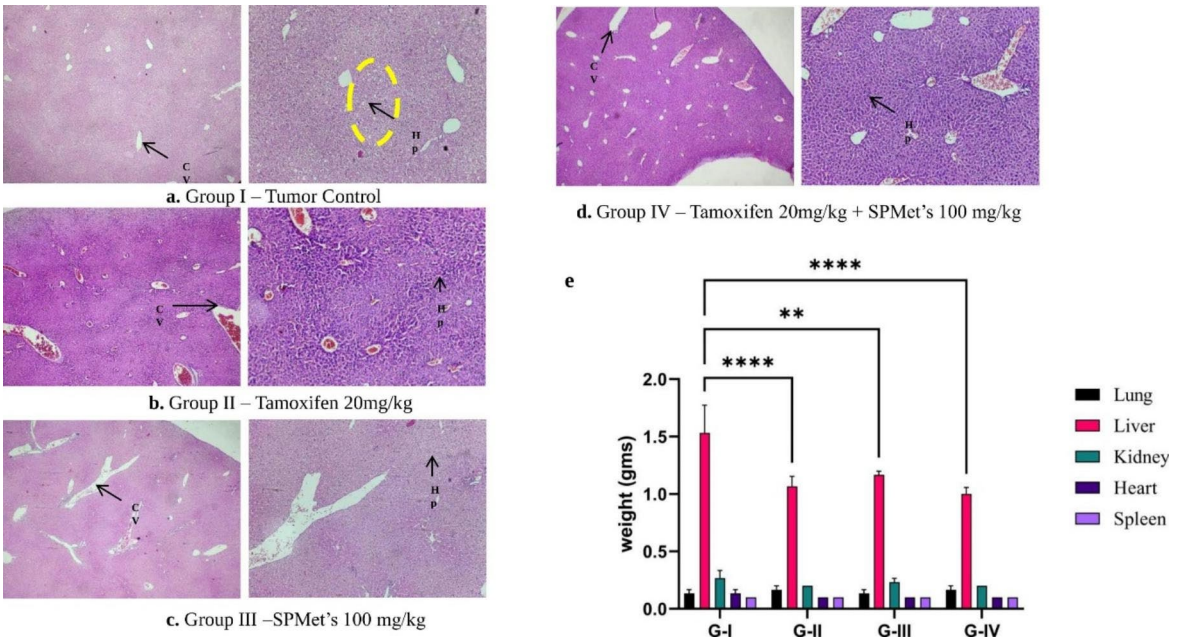

Fig. 3 **(a-d)** Microphotographs of organ sections of Liver; **(e)** Evaluation of organ weight. Values are represented as mean ± SD. ****p < 0.0001; **p < 0.01

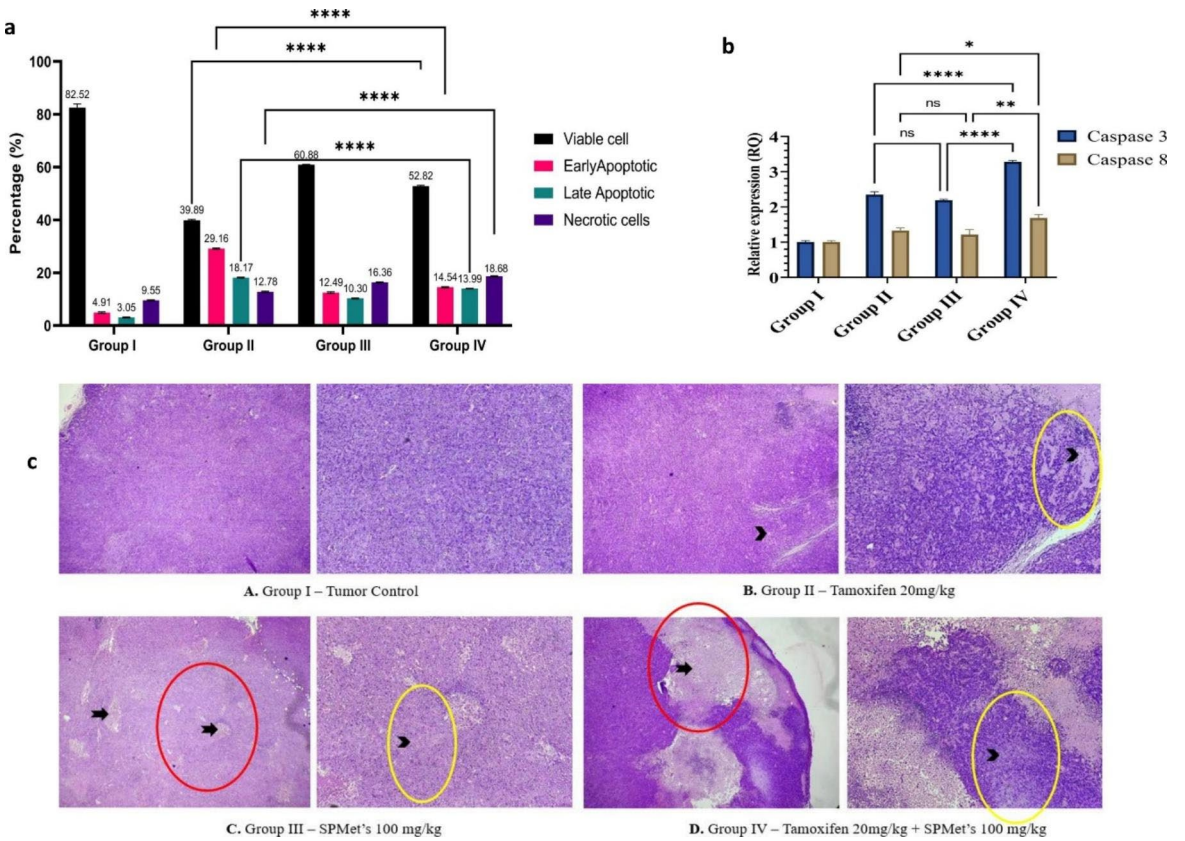

Fig. 4 **(a)** Apoptosis detection by FACS analysis; **(b)** Expression of Caspase 3 and 8 genes. Values are represented as mean ± SD. ****p < 0.0001; **p < 0.01; ns-p > 0.05; **(c)** Tumor Histopathology of all mice groups - Microphotographs of tumors to determine the apoptosis and necrosis caused during the treatment; Arrowhead – Apoptosis, Arrow- Necrosis

study, it was reported that long term administration of rMET resulted in 70% tumor reduction without any side effects, and inhibited the recurrence in patient-derived orthotopic xenograft (PDOX) mouse model with triple-negative breast cancer [28]. It can be well stated, through the current study outcomes, in line with some recent reviews [29], that the synergistic effects of natural compounds (L-Methioninase in this case) combined with chemotherapeutics might help to overcome some of the challenges encountered during breast cancer treatment.

Conclusion

It can be concluded through the current study results that the combination therapy with SPMet's and TAM is highly effective to address breast cancer when compared to monotherapy of TAM, in reducing tumor as well as keeping the mice healthy without major side effects. Tamoxifen citrate being the first-line chemotherapeutic agent for breast cancer, has not been tried in combination with L-Methioninase, hence this study report is the first one about the beneficial effects of semi purified Methioninase with TAM on breast cancer. These highly promising results open further avenues towards breast cancer treatment and the current combination has shown potential towards future clinical trials. However, since the small group size is the major limitation of the study, we feel it needs to be carried out with a larger group of animals before going for clinical trials.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-022-08144-z.

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Author contributions Conceptualization: Dr. KN Varalakshmi and Kavya D. Methodology: Dr. KN Varalakshmi and Kavya D. Validation: Dr. KN Varalakshmi. Formal analysis: Kavya D. Investigation: Kavya D. Writing – Original Draft: Kavya D. Writing – Review & Editing: Dr KN Varalakshmi and Kavya D. Supervision: Dr KN Varalakshmi. All authors read and approved the final manuscript.

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Data availability The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Statements & declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical statement This study was performed as per the guidelines of the Committee for the Purpose of Control And Supervision of Experiments on Animals (CPCSEA). This research was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) with IAEC approval no IAEC-SLS-2020-010. The animal experiment was carried out at Skanda life sciences Pvt. Ltd.

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