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Effect of Combination between Methotrexate and Histone Deacetylase Inhibitors on Transplantable Tumor Model

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Abstract Solid Ehrlich carcinoma is an undifferentiated tumor used in tumor studies. Methotrexate is an antimetabolite used in treatment of cancer, autoimmune diseases and induction of abortion. Valproic acid is used as anticonvulsant and is under investigation for treatment of cancer. The aim of this work was to study the effect of each of methotrexate and valproic acid alone and in combination on solid Ehrlich tumor in mice. Fifty albino mice were divided into five equal groups: Control untreated group, solid Ehrlich carcinoma, solid Ehrlich carcinoma + methotrexate, solid Ehrlich carcinoma + valproic acid, solid Ehrlich carcinoma + methotrexate + valproic acid. Tumor volume, tissue catalase, glutathione reductase, malondialdehyde, cholesterol and tumor necrosis factor-α were determined. A part of the tumor was examined for histopathological and immunohistochemical study. Methotrexate or valproic acid alone or in combination induced significant increase in tissue catalase and glutathione reductase with significant decrease in tumor volume, tissue malondialdehyde, cholesterol and tumor necrosis factor-α and alleviated the histopathological changes with significant increase in p53 expression and apoptotic index compared to solid Ehrlich carcinoma group. The combination of methotrexate and valproic acid has a better effect than each of methotrexate or valproic acid alone against solid Ehrlich tumor in mice.

Keywords: *methotrexate, histone deacetylase, tumor, mice*

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1. Introduction

Solid Ehrlich tumor is an undifferentiated solid tumor that is frequently used in tumor studies. It is both used to develop a tumor model and in chemotherapy investigations [1]. Following subcutaneous injection of Ehrlich tumor cells, a tumor of 1 cm in diameter is obtained approximately within one week. This highly virulent tumor causes the death of almost 100 % of experimental animals in a short period. Large-scale virulence, quick development and infiltrative nature of the tumor reflect its high-grade malignancy [2].

Methotrexate (MTX) is an antimetabolite and antifolate used in treatment of cancer, autoimmune and as an abortifacient in induction of medical abortion [3]. MTX competitively inhibits dihydrofolate reductase (DHFR), an enzyme that catalyses the conversion of dihydrofolate to the active tetrahydrofolate. Folic acid is needed for DNA synthesis. MTX, therefore, inhibits the synthesis of DNA, RNA and proteins. MTX is cytotoxic during the S-phase of the cell cycle. So, it has a greater toxic effect on rapidly dividing cells (such as malignant and myeloid cells, gastrointestinal and oral mucosa), which replicate their DNA more frequently and thus inhibits growth and proliferation of these cells as well as causing many side effects [4].

Valproic acid (VPA) is a chemical compound that has found clinical use as an anticonvulsant and mood-stabilizing drug, primarily in treatment of epilepsy, bipolar disorder, and, less commonly, major depression. It is also used to treat migraine headaches and schizophrenia [5]. Recently, VPA is under investigation for treatment of HIV and various cancers [6]. VPA not only suppresses tumor growth and metastasis, but also induces tumor differentiation in vitro and in vivo. VPA increases the DNA binding of activating protein-1 transcription factor, downregulates protein kinase C activity, inhibits glycogen synthase kinase-3b, activates the peroxisome proliferator-activated receptors and blocks HDAC (histone deacetylase), causing hyperacetylation. These findings elucidate an important role of VPA for cancer therapy [7]. The aim of this work is to study the effect of each of MTX and VPA alone and in combination on solid Ehrlich tumor in mice.

2. Materials and Methods

2.1. Drugs Used

- Methotrexate (Methotrexate 50 mg vial, Sanofi Aventis) was administered by intraperitoneal injection in a dose of 2.5 mg/kg body weight [8].
- Valproic acid (sodium salt; Sigma-Aldrich) was dissolved in saline and administered by intraperitoneal injection in a dose of 200 mg/kg body weight [9].

2.2. Solid Ehrlich Carcinoma (SEC) Tumor Model

A model of SEC was used, where $1X10^6$ of the Ehrlich carcinoma cells (ECC) obtained from the Pharmacology and Experimental Oncology Unit of the NCI, Cairo University, Egypt were implanted subcutaneously into the right thigh of the hind limb of mice. A palpable solid tumor mass (about $100mm^3$) was developed within 12 days [10].

2.3. Classification of Animals

In this study, we used 50 mice weighing about 20–25 g. All the experiments were conducted according to the National Research Council's guidelines. Animal handling was followed according to Helsenki declaration of animal ethics. The animals were divided into 5 equal groups of ten mice each as follows:

Group (1): is the control untreated group.

Group (2): Ehrlich tumor cells were implanted subcutaneously into the right thigh of the hind limb of mice [10].

Group (3): MTX was given by intraperitoneal injection on alternate days one week before and continued for 3 weeks after subcutaneous implantation of Ehrlich tumor cells into the right thigh of the hind limb.

Group (4): VPA was given by intraperitoneal injection once daily one week before and continued for 3 weeks after subcutaneous implantation of Ehrlich tumor cells into the right thigh of the hind limb.

Group (5): VPA and MTX were given by intraperitoneal injection one week before and continued for 3 weeks after subcutaneous implantation of Ehrlich tumor cells into the right thigh of the hind limb.

2.4. Assessment of the Effects of Different Treatments on Tumor Volume (TV) of SEC

Tumor volume was measured on days 12,14,16,18,20 and 22 after implantation of ECC cells using a Vernier caliper (Tricle Brand, Shanghai, China). The following formula was used to calculate the volume of the developed tumor mass [11]:

$$tumor\ volume(mm^3) = 4\pi\left(\frac{A}{2}\right)^2 \times \left(\frac{B}{2}\right)$$

Where A is the minor tumor axis; B the major tumor axis and π equals to 3.14.

At the end of the study, all mice were sacrificed. The tumor was excised and divided into two portions: one for homogenization and the other for histopathological and immunohistochemical examination. The tumor was homogenized for determination of tissue catalase according to the method described by Higgins et al. [12], tissue malondialdehyde according to Uchiyama & Mihara [13], tissue glutathione reductase according to the method of Manso & Wroblewski [14], tissue cholesterol using kits obtained from Biodiagnostic Co. according to the method of Richmond [15] and tissue tumor necrosis factor alpha (TNF-α) using mouse TNF-α ELISA kits supplied by RayBiotech, Inc. according to Luster & Rothenberg [16]. The SEC sections were prepared and stained with hematoxylin and eosin (H&E) and apoptotic indices were determined [17]. Immunohistochemical staining of p53 in formaline-fixed, paraffin embedded SEC sections was done, using Zymed's 2nd generation kit (Histostain®-Plus Kit) that utilizes the labeled streptavidin-biotin (LAB-SA) staining methodology (Zymed Laboratories Inc., Carlton Court, south San Francisco, CA.). Positive nuclei for p53 accumulation stained brown. The tumor was considered to be p53-positive if more than 10% of cells showed positive staining. The number of cells showing nuclear accumulation of p53 in positive tumors was expressed as follows: (++++): the largest number of cells showing positive nuclear staining for p53; (+++): intermediate number of p53-positive cells; (++): indicates lower number of cells with p53-stained nuclei [18].

3. Statistical Analysis

Data were presented as mean ± SEM, data were analyzed by one way analysis of normality of variance (ANOVA) using student t-test, differences between the means of different groups were considered significant at a level of $p< 0.05$.

Table 1. Apoptotic indices for SEC treated with MTX alone or VPA alone or their combination

Group	Apoptotic index (%)
SEC	1.42±0.21
SEC + MTX	4.71 ± 0.29*
SEC + VPA	3.62 ± 0.37*
SEC + MTX + VPA	5.21± 0.41*

Apoptotic index (%) was calculated for H&E stained sections of SEC excised on day 22 after implantation of ECC (values presented as the mean ± S.E.M. of an average of 10 fields)

* Indicates significant difference from SEC group at $P < 0.05$

4. Results

- Subcutaneous implantation of Ehrlich tumor cells into the right thigh of the hind limb of mice resulted in significant decrease in tissue catalase and tissue glutathione reductase with significant increase in tissue MDA, tissue cholesterol and tissue TNF-α compared to the control untreated group (Table 2, Figure 1).

- Intraperitoneal administration of MTX &/or VPA to mice resulted in significant increase in tissue catalase and tissue glutathione reductase with significant decrease in tumor volume, tissue MDA, tissue cholesterol and tissue TNF-α compared to the group that received subcutaneous implantation of Ehrlich tumor cells (Table 2, Figure 1).

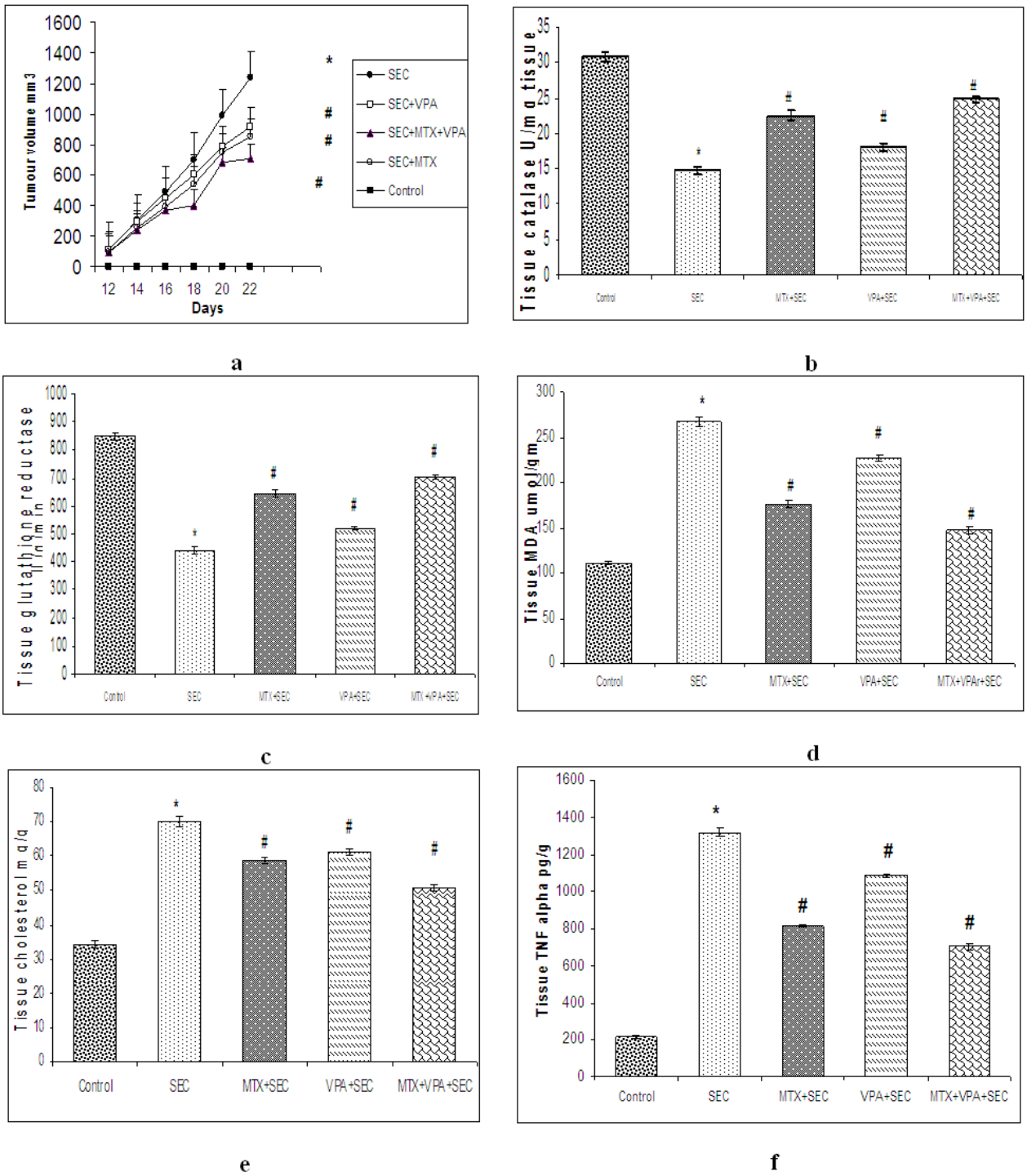

Figure 1. Effect of administration of MTX, VPA and their combination on tumor volume, tissue catalase, tissue MDA, tissue glutathione reductase, tissue cholesterol and tissue TNF-α in SEC treated mice
Values were represented as mean ± SEM. Number of mice in each group = 10
* Significant compared to the control group ($P < 0.05$)
# Significant compared to SEC group ($P < 0.05$)

Histopathological findings:

- Subcutaneous implantation of Ehrlich tumor cells resulted in development of Ehrlich solid tumor showing sheets of small, higher chromatophilic tumor cells of variable shape representing cell proliferation surrounding areas of necrosis and differentiated cells (Figure 2) with

negative staining for p53 (Figure 3). This picture was significantly improved in mice given MTX &/or VPA as evidenced by increasing degrees of necrosis (Figure 2), progressively increasing apoptotic indices (4.7 ± 0.29, 3.6 ± 0.37 and 5.1± 0.41% respectively) (Table 1) and increased expression of p53 (Figure 3).

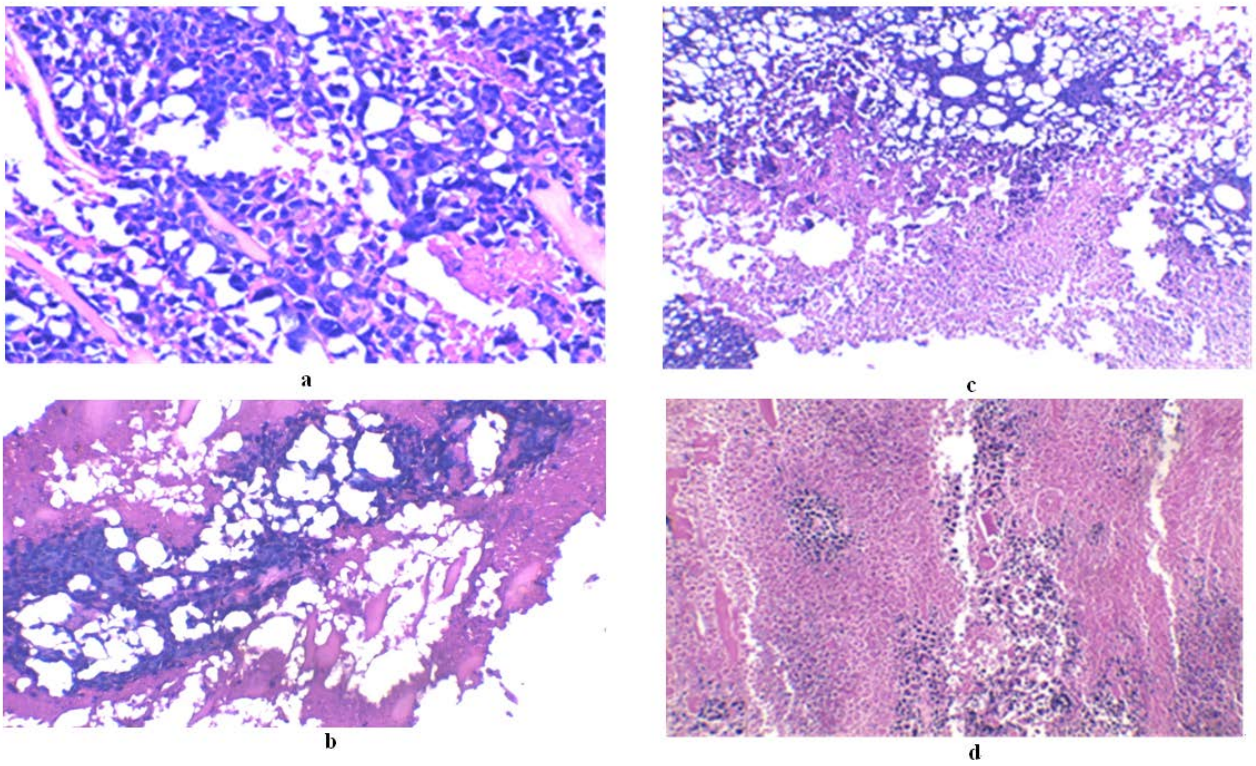

Figure 2. a: A photomicrograph of Ehrlich solid tumor showing sheets of small, higher chromatophilic tumor cells of variable shape representing cell proliferation surrounding areas of necrosis (H&E X 250). **b**: A photomicrograph of SEC sections from mice that received MTX showing sheets of malignant cells with focal necrosis and apoptosis (H&E X 250). **c**: A photomicrograph of SEC sections from mice that received VPA showing necrosis and apoptosis (H&E X 250). **d**: A photomicrograph of SEC sections from mice received MTX/VPA combination showing extensive necrosis and multiple apoptotic bodies (H&E X 250)

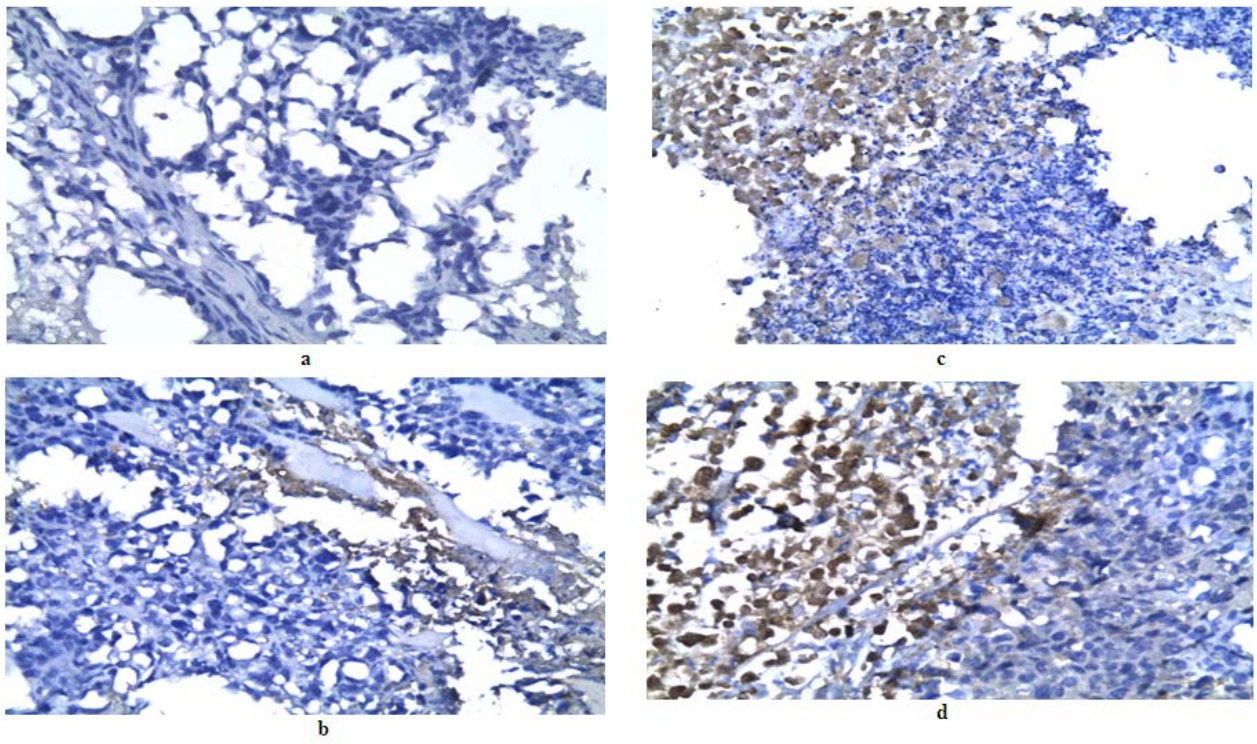

Figure 3. a: A photomicrograph of p53 staining of SEC sections from mice showing negative staining for p53 (PAP X 250). **b**: A photomicrograph of p53 staining of SEC sections from mice that received MTX showing positive (+++) p53 expression (PAP X 250). **c**: A photomicrograph of p53 staining of SEC sections from mice that received VPA showing positive (++) p53 expression (PAP X 250). **d**: A photomicrograph of p53 staining of SEC sections from mice that received MTX/VPA combination showing positive (++++) p53 expression (PAP X 250)

Table 2. Effect of different treatments on Tissue catalase, glutathione reductase, MDA, cholesterol and TNF-α in the studied groups

Group / Parameter	Group I Control	Group II SEC	Group III MTX+SEC	Group IV VPA+SEC	Group V MTX+VPA+SEC
Tissue catalase U/mg tissue	30.87±0.68	14.8±0.48*	22.49±0.73#	18.16±0.53#	24.82±0.42#
Tissue glutathione reductase U/g/min	848.3±10.4	440.4±10.39*	642.4±12.3#	520.21±8.16#	703.55±9.24#
Tissue MDA µmol/g tissue	110.82±2.6	266.51±5.34*	176.35±3.06#	227.22±3.25#	147.42±3.07#
Tissue cholesterol mg/g tissue	34.25±1.26	69.86±1.44*	58.68±0.87#	61.05±1.12#	50.72±0.9#
Tissue TNF-α pg/g tissue	216.01±6.75	1320.5±22.5*	813.65±9.03#	1088.65±9.76#	704.7±16.2#

Number of mice in each group = 10
Values expressed as mean ± SEM
* Significant compared to control group
# Significant compared to SEC group

5. Discussion

Ehrlich carcinoma is an undifferentiated carcinoma that is originally hyperdiploid, has high transplantable capability, no-regression, rapid proliferation, short life span, 100% malignancy and also does not have tumor-specific transplantation antigen. Ehrlich carcinoma has a resemblance with human tumors which are the most sensitive to chemotherapy due to the fact that it is undifferentiated and has a rapid growth rate [19]. Following implantation of Ehrlich tumor cells, morphological and metabolic changes occur such as structural deterioration, decreased number of mitochondria, decreased DNA and RNA synthesis, loss of intracellular purine and pyrimidine nucleotides, nucleosides and bases, a decline of ATP concentration and turnover, decreased protein synthesis, decreased glutathione concentration and increased triglycerides, cholesterol esters and free fatty acids [20].

In the present study, subcutaneous implantation of Ehrlich tumor cells into the right thigh of the hind limb of mice resulted in significant decrease in tissue catalase and tissue glutathione reductase with significant increase in tissue MDA, tissue cholesterol and tissue TNF-α compared to the normal control group. These results were in agreement with Badr El-Din [21] and Zahran et al. [22]. A number of studies had indicated that reactive oxygen species (ROS) are involved in a variety of different cellular processes ranging from apoptosis and necrosis to cell proliferation and carcinogenesis. In fact, molecular events, such as induction of cell proliferation, decreased apoptosis, and oxidative DNA damage have been proposed to be critically involved in carcinogenesis [23]. It was reported that during cancer growth, glutathione redox (GSH/GSSG) decreases in the blood of Ehrlich tumor–bearing mice. Glutathione reductase accounts for a very high GSH/GSSG ratio. The decrease in glutathione reductase activity in tissues correlated well with the decrease in G6PDH activity, decreased production of NADPH and the depletion of GSH content [24]. Cancer cells overexpress HMG-CoA reductase enzyme which is responsible for cholesterol synthesis. Ehrlich carcinoma had been characterized by increase cellular content of triglycerides and cholesterol esters [19]. The chemopreventive activity of statins against cancer is suggested to depend on inhibition of cholesterol synthesis and, thereby, cell growth [25]. TNF- α has been found to have a pro-cancerous effect. TNF-α produced by tumor cells or inflammatory cells in the tumor microenvironment can promote tumor cell survival through the induction of genes encoding nuclear factor-kB–dependent antiapoptotic molecules [26]. In a mouse skin model, TNF-α induces carcinogenesis. Furthermore, gene polymorphisms that increase or decrease TNF-α production confer either an increased risk or protective effect on a number of different cancers and precancerous diseases including gastric cancer, lymphoma and cervical cancer as well as cervical intraepithelial neoplasia. Moreover, in murine models, TNF-α promoted metastasis, tumor angiogenesis and cachexia. Trials with anti-TNF-α therapies are awaited to block this cytokine in patients with cancer [27].

Methotrexate (MTX) is an antimetabolite and antifolate drug which acts by inhibiting the metabolism of folic acid. It is known as a disease-modifying anti-rheumatic drug (DMARD), because it not only decreases pain and swelling of arthritis, but also can decrease damage to joints and long-term disability. It interferes with growth of certain body cells, especially cells that reproduce quickly, such as cancer cells, bone marrow cells and skin cells. It is used to treat certain types of cancer of the breast, skin, head and neck. It is also used to treat severe psoriasis [28].

In the present study, intraperitoneal administration of MTX to mice resulted in significant increase in tissue catalase and tissue glutathione reductase with significant decrease in tumor volume, tissue MDA, tissue cholesterol and tissue TNF-α and alleviated the histopathological changes with significant increase in the expression of p53 and apoptotic index compared to the group that received subcutaneous implantation of Ehrlich tumor cells. These results were in accordance with Srikanth et al. [8]. MTX is thought to affect cancer by two different pathways. It allosterically inhibits dihydrofolate reductase (DHFR), an enzyme that participates in the tetrahydrofolate synthesis. The affinity of MTX for DHFR is about one thousand-fold that of folate. DHFR catalyses the conversion of dihydrofolate to the active tetrahydrofolate. Folic acid is needed for the de novo synthesis of the nucleoside thymidine, required for DNA synthesis. Also, folate is needed for purine base synthesis, so all purine synthesis will be inhibited. So, MTX inhibits the synthesis of DNA, RNA, thymidylates and proteins. MTX acts specifically during DNA and RNA synthesis and so it is cytotoxic during the S-phase of the cell cycle [28]. Basak et al. [29] reported that the cytotoxic effect of MTX is associated with apoptosis enhancement, as it may be related to

hyperhomocysteinemia and deoxyribonucleotide pool imbalances. Moreover, Winter-Vann et al. [30] suggested that MTX has an inhibitiory effect on Ras signaling that regulates cell growth and differentiation. Because carboxyl methylation of Ras is important for proper plasma membrane localization and function, they reported that after MTX treatment, Ras methylation was decreased by almost 90% and subsequently inhibition of carboxyl methylation. Sung et al. [31] reported that MTX suppressed the interleukin-6 induced generation of ROS and so has a protective effect against tissue damage induced by oxidative stress. Moreover, MTX blocks tetrahydrofolate dependent steps in cell metabolism resulting in adenosine overproduction which inhibits TNF-α expression in a monocytic cell line and monocytes release adenosine after treatment with MTX [32]. Schnyder et al. [33] reported that application MTX to breast cancer produced significant decrease of total cholesterol which may be due to an inhibition of cholesterol synthesis depriving the tumor of the optimal amounts of cholesterol needed for cell division.

Valproic acid (VPA), a well known anticonvulsive agent, emerged as an antineoplastic agent as well, when findings indicated that VPA inhibited proliferation and induced differentiation of primitive neuroectodermal tumor cells in vivo [7]. In the present study, intraperitoneal administration of VPA to mice resulted in significant increase in tissue catalase and tissue glutathione reductase with significant decrease in tumor volume, tissue MDA, tissue cholesterol and tissue TNF-α and alleviated the histopathological changes with significant increase in the expression of p53 and apoptotic index compared to the group that received subcutaneous implantation of Ehrlich tumor cells.

The antitumor effects of VPA were attributed to that it is one of histone deacetylase (HDAC) inhibitors [7]. HDACs regulate the expression and activity of numerous proteins involved in both cancer initiation and cancer progression. HDACs bind to and deacetylate a variety of cellular proteins including transcription factors implicated in control of cell growth, differentiation and apoptosis [34]. It was suggested that VPA inhibits the proliferation of malignant tumor cells by a mechanism tightly coupled with HDAC. It was reported that VPA down-regulates HDAC-activity in neuroblastoma cells and so has antitumor activity [35].

Fourcade et al. [36] reported that VPA corrected the oxidative damage and decreased the levels of monounsaturated very long chain fatty acids and induced antioxidant effects in X-linked adrenoleukodystrophy. Glauben et al. [37] demonstrated that HDAC inhibitors including VPA suppressed cytokine production (Including TNF-α) and induced apoptosis and histone acetylation which reduced the risk of malignancies. VPA significantly suppressed mRNA levels of interferon-γ, TNF-α, IL-4, IL-6 and IL-17 in the lymph nodes and greatly attenuated accumulation of macrophages, T cells and B cells in experimental autoimmune neuritis [38].

Other studies reported that VPA is a potent inhibitor of tumor angiogenesis either directly by interfering with endothelial cells or indirectly by modulating tumor cells. VPA supressed the production of angiogenesis-related growth factors. Application of VPA to the colon adenocarcinoma not only led to a significant reduction of vascular endothelial growth factor (VEGF) secretion, but also to a down-regulation of protein expression as well as VEGF coding mRNA [39]. Moreover, VPA was found to modulate the MAP kinase pathway which plays an important role in the development of cancer, an effect that could be mediated through HDAC inhibition [40]. Additionally, Beger et al. [41] reported that VPA inhibited glycogen and RNA ribose turnover and disrupted glucose-derived cholesterol synthesis in mice which will deprive the tumor from cholesterol which is needed for tumor growth. Also, HDAC inhibitors were proven to reduce cholesterol accumulation in fibroblasts [42].

In the present study, intraperitoneal administration of MTX concomitantly with VPA resulted in significant increase in tissue catalase and tissue glutathione reductase with significant decrease in tumor volume, tissue MDA, tissue cholesterol and tissue TNF-α and alleviated the histopathological changes with significant increase in the expression of p53 and apoptotic index compared to the group that received subcutaneous implantation of Ehrlich tumor cells. This can be explained by the combined anti-inflammatory and antioxidant effect of MTX and VPA with their abilities to protect against the harmful effects of ROS, deprive the tumor of the optimal amounts of cholesterol needed for cell division and induction of apoptosis.

In conclusion, the present study demonstrated that the combination of MTX and VPA has a better effect than each of MTX or VPA alone against Ehrlich solid tumor in mice.

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