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ANTITUMOR AND ANTIANGIOGENIC EFFECTS OF THALIDOMIDE, ROFECOXIB, AND CAPTOPRIL IN EHRLICH ASCITES CARCINOMA IN MICE

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ABSTRACT

The study was conducted to evaluate the effect of thalidomide, rofecoxib and captopril on tumor growth and survival when used alone or in combination with cisplatin in Swiss albino mice. Solid tumors were induced by a subcutaneous injection of Ehrlich ascites carcinoma (EAC) cells. The antiangiogenic activity of these drugs was studied to explore the potential mechanism involved.

The EAC cells implanted subcutaneously to produce a solid tumor in the right flank of Swiss albino mice. This tumor was used to evaluate the anti-tumor and anti-angiogenic activities of thalidomide (100 mg/kg, i.p.), rofecoxib (20 mg/kg, p.o.) or captopril (50 mg/kg, p.o.) as individual treatments or in combination with cisplatin (2 mg/kg, i.p.). All treatments were started 24 hours after tumor cells inoculation. Tumor size was measured every other day for 21 days, tumor growth time (TGT) and tumor growth delay time (TGDT) were calculated. Animals were monitored and the mortality was recorded daily along the study period (100 days) to calculate the percentage survival of animals, mean survival time (MST) and percentage increased life span (%ILS). In a parallel experiment, the degree of angiogenesis was assessed by measuring the tumor vascular volume spectrophotometrically using 1% (w/v) Evan's blue.

Individual treatments with thalidomide, rofecoxib or captopril produced a significant ($p \leq 0.001$) reduction in tumor volume as compared to the control group. Their depressing effect on tumor volume tended to be enhanced progressively from the individual treatment to the combinations with cisplatin. The treatment with cisplatin, thalidomide and their combination could expand the life span of animals for 86, 96 and 100 days, respectively. Thalidomide and rofecoxib significantly ($p \leq 0.05$) inhibited the angiogenesis compared to the control group. The combination of cisplatin with thalidomide or rofecoxib further inhibited the angiogenesis significantly compared to the control group ($p \leq 0.001$) as well as cisplatin-treated group ($p \leq 0.05$). In the contrast, a significant inhibitory effect of captopril or cisplatin on angiogenesis was not evident. However, the combination of captopril and cisplatin could produce a significant inhibition of angiogenesis ($p \leq 0.001$).

These data indicate that thalidomide, rofecoxib, or captopril exerts an antitumorigenic effect on EAC solid tumor in Swiss mice. Thalidomide and rofecoxib proved to have an antiangiogenic effect in EAC-model. Our results suggest the use of antiangiogenic agents as an adjuvant treatment to chemotherapy.

INTRODUCTION

One of the most promising areas of cancer research involves inhibition of blood vessel growth, called angiogenesis[1]. Tumor angiogenesis is a critical process for tumor growth and it serves as a venue for tumor cell dissemination and metastasis. Growth of tumor beyond few millimeters requires the induction of new capillary blood vessels[2]. Several clinical reports demonstrated a significant correlation between the degree of tumor vascularity and the patient's prognosis in almost all solid tumors[3,4]. Therefore the notion has emerged that tumor growth could be restricted by selectively inhibiting the angiogenic process with proper antiangiogenic drugs that exert fewer inhibitory effects on the proliferation of host cells[5].

Thalidomide (*α-N*-phthalimidoglutarimide) was introduced in the 1950s as a safe sedative with a remarkable antiemetic effect. After the reports about thalidomide's teratogenicity early in the 1960s[6,7], this drug was withdrawn from the market, but recently renewed interest has gained because of its diverse pharmacologic effects other than the teratogenicity[8].

Thalidomide emerged as an antiangiogenic drug from the assumption that the dysmelia in newborn thalidomide babies might be related to the inhibition in vasculogenesis during the early gestational period[9]. Since then an experimental report established a strong antiangiogenic effect of thalidomide in an angiogenic growth factor-induced angiogenesis model[10], but the antiangiogenic effect of this drug in tumor angiogenesis is yet to be established.

Prostaglandins are implicated in the development and growth of malignant tumors[11]. Cyclooxygenase (COX), the key regulatory enzyme for prostaglandin synthesis, is transcribed from two distinct genes. COX-I is expressed constitutively in most tissues whereas COX-II is rapidly induced at sites of inflammation and at sites of proliferation, for example within tumors[12,13]. Increasing evidence shows that NSAIDs can inhibit tumor growth in experimental animals and in humans[14]. Commonly used NSAIDS, such as indomethacin, inhibit both COX-I and COX-II but treatment with such agents may be limited by toxicity to normal tissues, particularly ulceration and bleeding in the gastrointestinal tract as a consequence of COX-I inhibition[14]. Selective COX-II inhibitors, for example rofecoxib, have been shown to prevent carcinogenesis in experimental models of intestinal and colon cancer[15,16] and chemically-induced breast cancer[17]. The possible mechanism of anti-tumor effects of COX-II inhibitors may be due to inhibition of angiogenesis[18] or induction of apoptosis[19].

Captopril (D-3-mercapto-2-methylpropanoyl-L-proline), an inhibitor of the angiotensin-converting enzyme, is widely used in the treatment of several cardiovascular diseases[20]. In addition, captopril has a

number of other biological activities[21]. It can ameliorate arthritis[22], reverse diabetic retinopathy[23], enhance insulin sensitivity[21], lower thrombotic risk[20], decrease atherosclerosis and renal failure[24,25], and lower the incidence of radiation-induced pulmonary damage and radiation-induced fibrosarcomas in rats[26].

Several of the diseases that respond favorably to captopril, including arthritis, diabetic retinopathy, atherosclerosis and cancer are angiogenesis dependent[27], raising the possibility that captopril could be limiting their progression in part by inhibiting the new blood vessel growth on which their pathology depends[28]. Indeed, captopril inhibits endothelial-cell migration by blocking the activity of Zn^{2+} dependent metalloproteinases required by endothelial cells to respond to angiogenic stimuli[28]. Agents that inhibit angiogenesis and extracellular-matrix degradation may complement other anti-tumor therapies, such as chemotherapy, to further inhibit tumor growth and metastatic spread[29].

The efficacy of *cis*-dichlorodiammineplatinum (II), marked as cisplatin, has been thoroughly studied since its introduction to cancer therapy[30]. Cisplatin has proved to be effective against solid tumors of the cervix, bladder, and prostate[31]. The cytotoxicity of cisplatin lies in its ability to form interstrand and intrastrand cross-links in DNA[30]. Chemoprevention is a promising strategy to inhibit carcinoma before invasive tumors develop, but it is far from being satisfactory, especially because of its significant toxicity. Hence, new molecular targets are needed[32]. The concept of supplementing chemotherapy with antiangiogenic agents is aimed at improving the effect of chemotherapy without increasing the toxicity to the patient[33].

The purpose of the present study was to evaluate the effect of thalidomide, rofecoxib or captopril on tumor growth and angiogenesis, when used alone and in combination with cisplatin in Swiss albino mice whose solid tumors were induced by a subcutaneous injection of Ehrlich ascites carcinoma (EAC) cells.

MATERIALS AND METHODS

Animals:

Female Swiss albino mice weighing 20-25 g, obtained from the Egyptian Organization for Biological Products and Vaccines (Vacsera, Egypt), were used in this study. Animals were kept at 25°C on a 12-hours dark/light cycle. The mice were housed in plastic cages (40×30×17cm) where hardwood bedding was used. Ten animals were kept per cage. Food and water were allowed *ad libitum*.

Tumor cells:

Ehrlich Ascites Carcinoma (EAC) cell line was purchased from Tumor Biology Department, National Cancer Institute, Cairo University. EAC is a murine spontaneous breast cancer that served as the original tumor from which an ascites variant was obtained. On intraperitoneal inoculation, an ascites rich in tumor cells is produced. The tumor cell line was maintained in female Swiss albino mice by serial i.p. passage in female Swiss albino mice at 7-10 days interval. The EAC cells were prepared under aseptic conditions. EAC cells was tested for viability and contamination using Trypan blue dye exclusion technique[34], where this dye stains dead cells only. EAC cells were suspended in normal saline so that each 0.1 ml contained 2.5×10^6 cells. Cells were counted under the microscope using Neubauer haemocytometer.

Drugs:

Cisplatin

It is supplied as dry lyophilized powder in vials (Platinol, Bristol Myers Squibb Co., USA). In the current study a dose of 2 mg/kg body weight, i.p., was used[35]. The contents were freshly dissolved in sterile water for injection.

(±)-Thalidomide

The powder was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Thalidomide was freshly prepared on each treatment day. It was first emulsified in 0.1 ml Tween-80 and then suspended in phosphate buffered saline (PBS) to reach a final concentration such that an injection of 0.1 ml, i.p., would deliver 100 mg/kg body weight[36].

Rofecoxib

Supplied as an oral suspension (Selox, Medical Union Pharmaceuticals (MUP), Ismailia, Egypt). In the current study a dose of 20 mg/kg body weight, p. o., was used[16].

Captopril

The powder was obtained from Pharaonia Pharmaceuticals, Alexandria, Egypt. In the current study a dose of 50 mg/kg, p.o., was used[37].

Study design and methodology

The whole study was divided into two major experiments:

I- Antitumor activity (Tumor development & survival study).

II- Assessment of the angiostatic activity.

I- Anti-tumor activity

This experiment was conducted to assess the anti-tumor effect of cisplatin alone and in combination with thalidomide, rofecoxib, or captopril using EAC cells implanted as a solid tumor in the right flank of female Swiss albino mice.

Eighty female Swiss albino mice were used in this experiment. Each mouse was inoculated s.c. in the right flank with 2.5×10^6 EAC cells in 0.1 ml saline. Twenty-four hours after tumor inoculation, the animals were randomly divided into 8 groups, 10 animals each. Table 1 summarizes various groups and their treatment regimens.

Table 1: Summery of the study design.

Groups	Treatment regimen
Control	Saline, 0.1ml, i.p.
Cisplatin*	Cisplatin, 2mg/kg, i.p.
Thalidomide	Thalidomide, 100mg/kg, i.p.
Rofecoxib	Rofecoxib, 20mg/kg, p.o.
Captopril	Captopril, 50mg/kg, p.o.
Cisplatin+Thalidomide	Cisplatin, 2mg/kg, i.p. + Thalidomide, 100mg/kg, i.p.
Cisplatin+Rofecoxib	Cisplatin, 2mg/kg, i.p. + Rofecoxib, 20mg/kg, p.o.
Cisplatin+Captopril	Cisplatin, 2mg/kg, i.p. + Captopril, 50mg/kg, p.o.

All treatments (except cisplatin) were given for 21 consecutive days starting 24 hours after tumor cells inoculation.

* Cisplatin was given for 3 consecutive days[35] starting 24 hours after tumor cells inoculation.

Five days after tumor inoculation, when the tumor became palpable, the initial tumor volume of each animal was measured using Vernier Caliper (Optilab, Berlin, Germany). The tumor volume for each animal was measured every other day along the experiment. Tumor growth time (TGT) and tumor growth delay time (TGDT) were calculated.

Animals were monitored and the mortality was recorded daily along the study period (100 days) to calculate the percentage survival of animals, mean survival time (MST) and percentage increased life span (%ILS). The study period was determined by the longest life span among all animals.

The tumor volume: The tumor volume was calculated according to the following formula:

Tumor volume (mm^3) = $0.5\,A^2\,B$

Where A and B are the minor and major tumor axes, respectively[37].

Tumor growth time (TGT): It was calculated as the average days required by the tumor to reach double, triple or quadruple the initial tumor volume[38].

Tumor growth delay time (TGDT): It was calculated as the difference between the average tumor growth time of the treatment group (TGTt) and the control group (TGTc) to the double, triple or quadruple the initial tumor volume.

Percentage survival of animals = [number of living mice/initial total number of mice (10)] ×100.

Mean survival time (MST) = Sum of survival days of all mice in each group/ total number of mice in this group[10].

Percentage increased life span (%ILS) = [(Mean survival time of treated group / Mean survival time of control group) – 1] × 100.

An enhancement of life span by 25% or more was considered as effective anti-tumor response[39].

II- Angiostatic activity:

Assessment of the angiostatic activities of cisplatin alone and in combination with thalidomide, rofecoxib or captopril on EAC-bearing female Swiss albino mice was done according to the method of Lee *et al.*[40].

Eighty female Swiss albino mice were used. Each mouse was inoculated i.d. at 4 sites bilaterally on the lower ventral side (after shaving this area) with 100 µl EAC suspension (2.5×106 cells/ 0.1 ml) on each site. Animals were randomly divided into 8 groups. The treatment was initiated 24 hours after tumor inoculation, which was designated day (0). Animals were given the drug regimens, mentioned under the anti-tumor experiment section, for 3 consecutive days.

The degree of angiogenesis was assessed by measuring the tumor vascular volume on day 3 as follows:

Twenty-four hours after administration of the last dose, each mouse was injected i.v. with 0.25 ml (1% w/v) Evan's blue (Sigma, USA) through the tail vein. Two minutes after dye injection each mouse was killed by cervical dislocation and implantation sites were punched out. Each two skin discs were pooled up in 2 ml standard solution (mentioned below) and kept at room temperature for 24 hours with occasional shaking. Following centrifugation, at 4000 rpm for 5 minutes, the absorbance of the supernatant was measured at 620 nm using spectrophotometer.

Standard solution was prepared by injecting two normal mice (i.v.) through the tail vein with 0.25 ml of 1% (w/v) Evan's blue. Two minutes thereafter, the blood was withdrawn from orbital sinus under light ether anesthesia using heparinized microcapillaries and layered on a solution of sodium sulfate/acetone (0.5% Na2SO4, acetone=2/3 v/v) at a concentration of 2 µl blood/ml. The suspension was kept at 4°C for 24 hours. The supernatant (the standard solution) was then separated by centrifugation at 4000 rpm for 5 minutes and stored at 4°C until use.

The results were expressed as percentage of angiogenesis which was calculated according to the following formula:

$$\%\ \text{Ang.} = [(A-B)/(C-B)] \times 100$$

Where A, B and C represent the optical density measured at 620 nm of the treated tumor, background and control tumor, respectively.

Statistical analysis

Data were computed as the mean ± standard error of the mean and compared using SPSS (version 9). Multiple comparisons were carried out using one way analysis of variance (ANOVA) followed by Bonferroni test for selected pairs. Chi square test was used to analyze the effect of different treatments on survival.

RESULTS

I- Anti-tumor activity

In the current study, the anti-tumor effect of cisplatin alone and in combination with thalidomide, rofecoxib, or captopril was assessed by change in

tumor volume, tumor growth time (TGT), tumor growth delay time (TGDT), percent survival of animals, mean survival time (MST) and percentage increased life span (%ILS).

Effect on tumor volume

Treatment with cisplatin (2 mg/kg, i.p.) for 3 consecutive days significantly ($p \leq 0.001$) reduced the tumor volume compared to the control group (Figure 1).

The administration of thalidomide (100 mg/kg, i.p.) daily for 21 days also significantly ($p \leq 0.001$) reduced the tumor volume to a lesser extent than cisplatin. The combination of thalidomide with cisplatin produced a further significant reduction in the tumor volume compared to the control group ($p \leq 0.001$) (Figure 1).

Similarly, rofecoxib (20 mg/kg, p.o.) or captopril (50 mg/kg, p.o.) and their combinations with cisplatin produced a significant ($p \leq 0.001$) reduction in tumor volume as compared to the control group (Figures 2 and 3).

Although there was no significant difference between individual treatments and their combination with cisplatin, their depressing effect on tumor volume tended to be enhanced progressively from the individual treatment to the combinations with cisplatin (Figures 1-3).

Effect on tumor growth time (TGT)

Administration of cisplatin (2 mg/kg, i.p.) for 3 consecutive days failed to significantly prolong TGT; however, cisplatin tended to increase TGT required to reach 2-, 3-, and 4-fold the initial tumor volume from the 5th day of inoculation by 124±3.4%, 137±3.7% and 137±3.02%, respectively, as compared to the control group (Figure 4).

Although the individual treatments of thalidomide, rofecoxib or captopril didn't show any significant effect on TGT, their combinations with cisplatin produced a significant increase in TGT as compared to the control group ($p \leq 0.001$). The combination of thalidomide with cisplatin could produce a significant increase in TGT required to reach 2-, 3-, and 4-fold the initial tumor volume from the 5th day of inoculation by 280±40.9%, 306±15.2% and 309±9.3%, respectively ($p \leq 0.001$) (Figure 4). A significant difference was also found between the individual treatment of thalidomide and its combination with cisplatin ($p \leq 0.001$) (Figure 4).

In the same pattern, the co-administration of rofecoxib with cisplatin significantly ($p \leq 0.001$) increased TGT by 332±32.5%, 322±20.5% and 317±14.5%, respectively, compared to the control group (Figure 4). There was also a significant difference between the combination group and the group treated with rofecoxib alone ($p \leq 0.001$) (Figure 4).

The treatment with captopril in combination with cisplatin significantly ($p \leq 0.001$) increased TGT by 250±26.9%, 266±26.2% and 262±24.8%, respectively, compared to the control group (Figure 4). There was also a significant difference between the combination treatment and the captopril individual treatment ($p \leq 0.05$) (Figure 4).

Effect on tumor growth delay time (TGDT)

The tumor growth delay time (TGDT) values were calculated as the difference between the average tumor growth time of the treatment group (TGTt) and the control group (TGTc) to the double, triple or quadruple the initial tumor volume. Following cisplatin (2 mg/kg, i.p.) administration, TGDT values were 2.4±0.34, 2.6±0.46 and 5.47±0.48 days, respectively.

The combination of thalidomide with cisplatin significantly ($p \leq 0.001$) increased TGDT values to reach 2-, 3-, and 4-fold the initial tumor volume by 758±171.9%, 1084±41.4% and 542±24.1%, respectively, compared to cisplatin (Figure 5).

Similarly, the co-administration of rofecoxib with cisplatin significantly ($p \leq 0.001$) increased TGDT to reach double, triple or quadruple the initial tumor volume by 982±154.2%, 1069±60.3% and 564±31.6%, respectively, compared to cisplatin (Figure5).

The treatment with captopril in combination with cisplatin significantly ($p \leq 0.001$) increased TGDT values to reach 2-, 3-, and 4-fold the initial tumor volume by 633±89.2%, 800±53.4% and 422±44.1%. respectively, compared to cisplatin (Figure 5).

Effect on percent survival of animals

The change in percent of survival of animals was recorded daily for a period of 100 days following tumor inoculation.

On day 58, there was a 50% survival in the control group, whereas, cisplatin, thalidomide and their combination maintained 100% survival (Figure 6. upper panel). Other combinations of cisplatin with rofecoxib or captopril could maintain 80% and 90% survival, respectively (Figure 6, middle and lower panels).

The control EAC-bearing mice showed Zero % survival after 79 days of the experiment. On the same day, a 30% and 50% survival was noted in the cisplatin and thalidomide group, respectively. The combination treatment of these drugs could successfully maintain 90% survival (Figure 6).

Rofecoxib and its combination with cisplatin produced 30% survival on day 79. While captopril and its combination with cisplatin maintained the least survival (10%) (Figure 6). The treatment with cisplatin, thalidomide and their combination could expand the life span of animals for 86, 96 and 100 days, respectively (Figure 6).

Effect on mean survival time (MST)

Mean survival time (MST) was calculated as the sum of survival days of all mice in each group divided by the total number of mice in this group. In the EAC

control group, the mean survival time was 50±6.75 days. Although the reference substance (cisplatin) tended to increase MST to reach 73.4±2.67 days, it failed to produce a significant difference from the control. In contrast, thalidomide significantly ($p \leq 0.01$) increased MST to 78.6±3.55 days. Furthermore, the combination of thalidomide with cisplatin could produce the highest increase in MST (89.9±2.48) which was significantly ($p \leq 0.01$) different from the control (Table 2). All other treatments did not show significant changes in MST values.

Effect on life span (%ILS)

Parallel to the results of MST, thalidomide and its combination with cisplatin could produce the highest %ILS, 57.2% and 79.8%, respectively, compared to the control group (Table 2). All other treatments, except captopril, could produce an enhancement of the life span by more than 25% compared to the control group (Table 2).

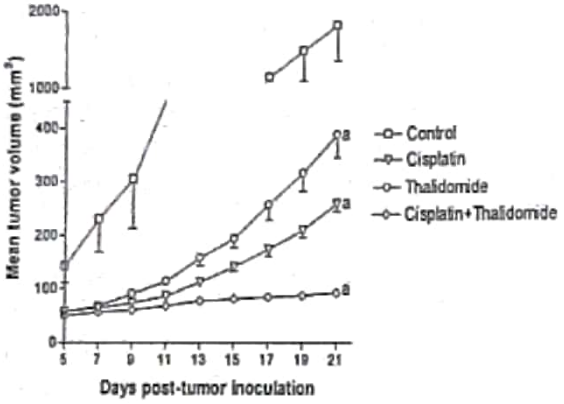


Figure 1. Effect of cisplatin (2 mg/kg, i.p.) and/or thalidomide (100 mg/kg, i.p.) on the tumor volume of EAC cells injected s.c. (2.5×10^6 cells/mouse).
Values are expressed as the mean volume of tumor ± SE.
a: Significantly different from control at $p \leq 0.001$.
Number of animals in each group (n) = 10.

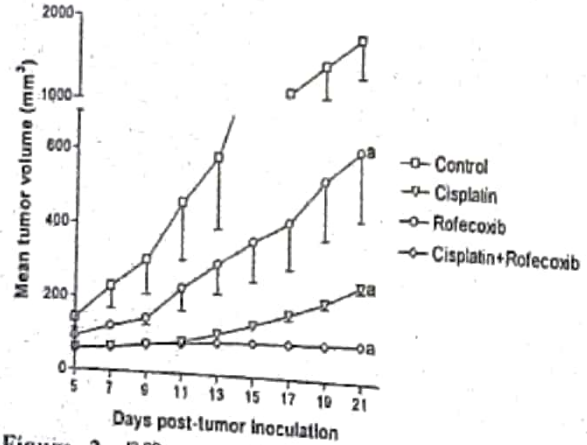


Figure 2. Effect of cisplatin (2 mg/kg, i.p.) and/or rofecoxib (20 mg/kg, p.o.) on the tumor volume of EAC cells injected s.c. (2.5×10^6 cells/mouse).
Values are expressed as the mean volume of tumor ± SE.
a: Significantly different from control at $p \leq 0.001$.
Number of animals in each group (n) = 10.

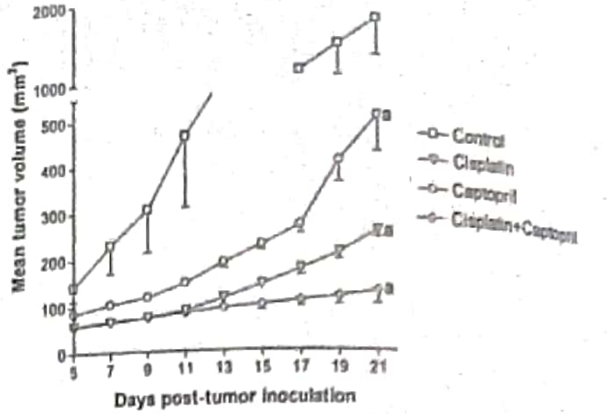


Figure 3. Effect of cisplatin (2 mg/kg, i.p.) and/or captopril (50 mg/kg, p.o.) on the tumor volume of EAC cells injected s.c. (2.5×10^6 cells/mouse) in female Swiss albino mice.
Values are expressed as the mean volume of tumor ± SE.
a: Significantly different from control at $p \leq 0.001$.
Number of animals in each group (n) = 10.

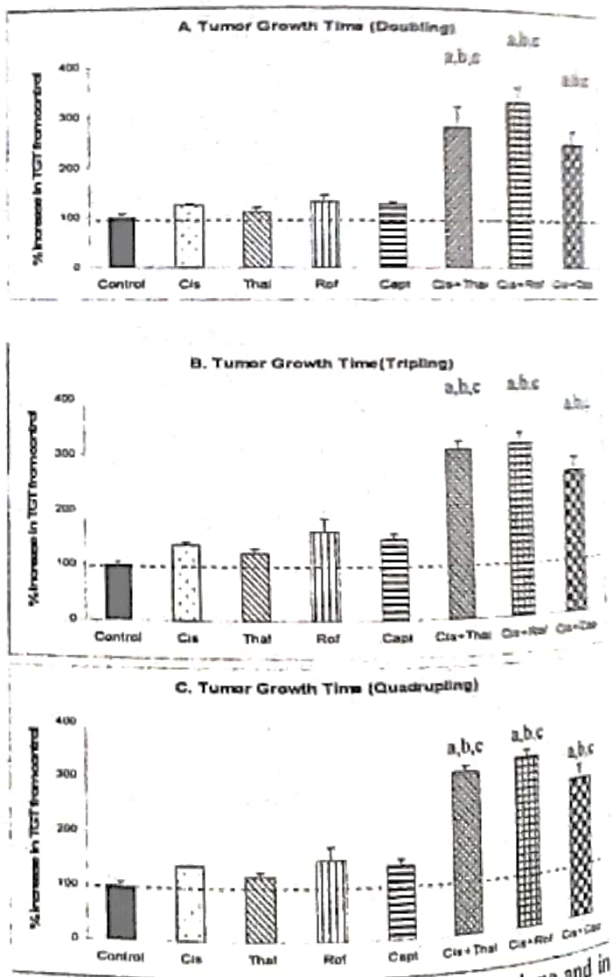


Figure 4: Effect of cisplatin (2 mg/kg, i.p.) alone and in combination with thalidomide (100 mg/kg, i.p.), rofecoxib (20 mg/kg, p.o.) or captopril (50 mg/kg, p.o.) on the percentage increase in tumor growth time (TGT) from control to reach double (A.), triple (B.) or quadruple (C.) the initial tumor volume in EAC-bearing female Swiss albino mice. a: Significantly different from control at $p \leq 0.001$. b: Significantly different from cisplatin at $p \leq 0.001$. c: Significantly different from individual corresponding treatment (thalidomide, rofecoxib or captopril) at $p \leq 0.05$.
Number of animals in each group (n) = 10.

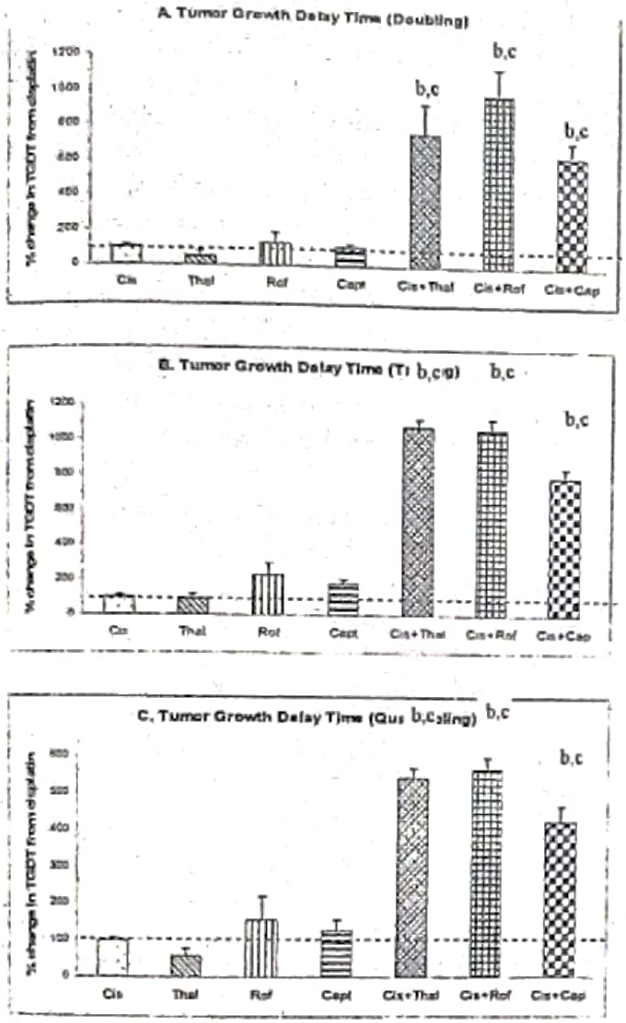
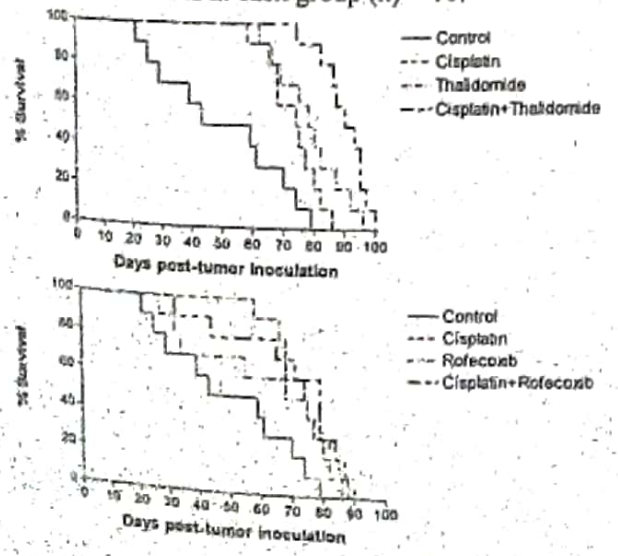


Figure 5: Effect of cisplatin (2 mg/kg, i.p.) alone and in combination with thalidomide (100 mg/kg, i.p.), rofecoxib (20 mg/kg, p.o.) or captopril (50 mg/kg, p.o.) on the percentage increase in tumor growth delay time (TGDT) from cisplatin to reach double (A.), triple (B.) or quadruple (C.) the initial tumor volume in EAC-bearing female Swiss albino mice.
b. Significantly different from cisplatin at $p \leq 0.001$, c: Significantly different from individual corresponding treatment (thalidomide, rofecoxib or captopril) at $p \leq 0.001$.
Number of animals in each group (n) = 10.



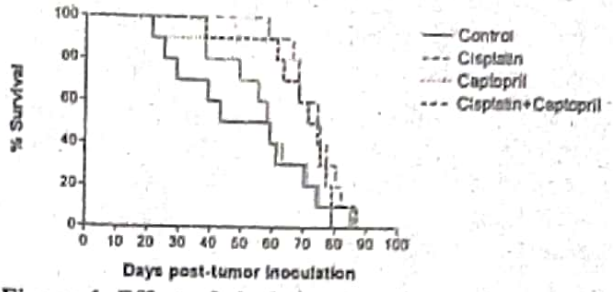


Figure 6. Effect of cisplatin (2 mg/kg, i.p.) alone and in combination with thalidomide (100 mg/kg, i.p.), upper panel, rofecoxib (20 mg/kg, p.o.), middle panel, or captopril (50 mg/kg, p.o.), lower panel, on percent survival of EAC-bearing mice. Number of animals in each group (n) = 10.

Table 2: Effect of cisplatin (2 mg/kg, i.p.) alone and in combination with thalidomide (100 mg/kg, i.p.), rofecoxib (20 mg/kg, p.o.) or captopril (50 mg/kg, p.o.) on mean survival time of EAC-bearing female Swiss albino mice.

Group	MST* (days)	% ILS**
Control	50.0±6.57	
Cisplatin	73.4±2.67	46.8
Thalidomide	78.6±3.55[a]	57.2
Rofecoxib	62.7±7.57	25.4
Captopril	57.4± 5.84	14.8
Cisplatin+Thalidomide	89.9±2.48[a]	79.8
Cisplatin+Rofecoxib	70.8±5.96	41.6
Cisplatin+Captopril	69.3±4.23	38.6

* Mean Survival Time (MST) = sum of survival days of all mice in each group/total number of mice in this group (10).
** % Increase in Life Span (%ILS) = [(MST of treated group/MST of control group)-1] x100.
Values are expressed as mean ± SE.
a: Significantly different from control at $p \leq 0.01$.
Number of animals in each group (n) = 10.

II- Angiostatic Activity

In this experiment, the angiostatic activities of cisplatin alone and in combination with thalidomide, rofecoxib or captopril on EAC-bearing female Swiss albino mice were investigated. The degree of angiogenesis was assessed by measuring the tumor vascular volume spectrophotometrically using 1% (w/v) Evan's blue.

As deduced from the angiogenesis equation, tumor inoculation induced 100% angiogenesis in the control group, which represented a base line for angiogenesis. Administration of cisplatin (2 mg/kg, i.p.) for 3 consecutive days failed to produce a significant reduction in angiogenesis. In the contrast, thalidomide (100 mg/kg, i.p.) significantly ($p \leq 0.05$) inhibited the angiogenesis to 66.6±4.8% compared to the control group. The combination of thalidomide and cisplatin further inhibited the angiogenesis significantly by

50% compared to the control group ($p \leq 0.001$) as well as cisplatin-treated group ($p \leq 0.01$) (Table 3).

Similarly, rofecoxib (20 mg/kg, p.o.) significantly ($p \leq 0.05$) reduced the angiogenesis to reach 56.02±8.99%. Furthermore, the combination of rofecoxib and cisplatin produced the maximal reduction in angiogenesis to reach 42.8±4.09%, which was significantly different from both the control group ($p \leq 0.001$) and cisplatin-treated group ($p \leq 0.01$) (Table 3).

Although the administration of captopril (50 mg/kg, p.o.) couldn't show any significant inhibition of angiogenesis, its combination with cisplatin significantly ($p \leq 0.001$) inhibited the angiogenesis to reach 55.97±5.3% compared to the control group (Table 3).

Table 3: Effect of cisplatin (2 mg/kg, i.p.) alone and in combination with thalidomide (100 mg/kg, i.p.), rofecoxib (20 mg/kg, p.o.) or captopril (50 mg/kg, p.o.) on the percentage of angiogenesis in EAC-bearing female Swiss albino mice.

Group	*Percentage of angiogenesis*
Control (n=6)	100±8.4
Cisplatin (n=7)	84.65±1.5
Thalidomide (n=8)	66.6±4.8[a]
Rofecoxib (n=8)	56.02±8.99[a]
Captopril (n=7)	69.3 ±8.5
Cisplatin+Thalidomide (n=6)	50.2±5.9[a,b]
Cisplatin+Rofecoxib (n=6)	42.8±4.09[a,b]
Cisplatin+Captopril (n=6)	55.97±5.3[a]

Values are expressed as mean ± SE.
a: Significantly different from control at $p \leq 0.001$. b: Significantly different from cisplatin at $p \leq 0.001$.
n is number of animals in each group.

DISCUSSION

Tumor angiogenesis is a critical step for the growth and metastasis of solid tumors[41]. Several antiangiogenic drugs have been developed with promising results in experimental settings[38]. The antiangiogenic effect of thalidomide, rofecoxib and captopril was investigated in the current study.

Unsuccessful studies utilizing thalidomide as a single agent in both mice and humans have recommended that future research be done to evaluate its efficacy as an adjuvant to chemotherapy[42,43]. The current study has documented the ability of the individual treatment of thalidomide to reduce solid tumor growth in Swiss mice. There have been conflicting results as to anti-tumor and anti-angiogenic effects of thalidomide. It was shown that thalidomide inhibited both basic fibroblast growth factor (bFGF) - and vascular endothelial growth factor (VEGF)-induced corneal neovascularization[10] and TNF-α mRNA expression[44]. The inhibition of tumor growth by 55% in the rabbit oral carcinoma model by thalidomide alone has been reported[45]. Others failed to demonstrate similar inhibitory effects[46,47]. In a phase II clinical study using thalidomide, a few patients with renal cancers showed some response, but no objective responses were seen in ovarian, breast cancers, and melanomas[41]. When murine breast cancer was treated with thalidomide and chemotherapy drugs, reduced primary and secondary tumor growth were recorded[42].

In the current study, the combination of thalidomide and cisplatin seems to be superior to thalidomide alone. There was no significant difference between both treatment regimens.

It seems that the timing of treatment on the course of tumor progression is critical. A previous study, which withheld treatment until tumor formation, reported the inability of thalidomide to inhibit solid tumor formation[42]. Whereas, the same authors showed a depressing effect of thalidomide on tumor volume when treatment was initiated before the tumor volume reached 50 mm^3[42]. Therefore, in the present study, treatment with thalidomide was commenced 24 hours after cell inoculation.

COX-II inhibitors are another proposed antiangiogenic agents. To the best of our knowledge there are no studies determining the efficacy of COX-II inhibitors in the chemoprevention of Ehrlich ascites carcinoma. In the current study, we observed such efficacy by evaluating rofecoxib inhibition of EAC-induced tumor.

It has been known that induction of COX-II enzyme is an early event in the development of carcinogenesis[48]. This fact implies that this enzyme is an excellent target for early intervention and supports the rationale of using rofecoxib for cancer chemoprevention before invasive tumors develop.

In recent studies, it was found that COX-II enzyme is not normally present under physiological conditions in tissues but is extensively expressed in cancer, especially in cancers with a high angiogenic activity. The enzyme is rapidly induced in most cancer tissues including colon, lung, breast, and prostate[32]. In a clinical study, immunohistochemical analysis of tissue samples collected from patients showed that COX-II was extensively expressed in oral and other head and neck cancer[49]. Interestingly, there is evidence to indicate that the expression of COX-II in cancer was mainly restricted to the new blood vessels, the preexisting vasculature adjacent to the primary tumor, and the blood vessels invading the metastatic lesion, however, the expression was not present in the tumor cells themselves[50]. All findings suggest that COX-II may be an excellent target for the development of new strategies in the prevention or treatment of cancers. In the current study, our findings support the hypothesis about the effect of rofecoxib on tumor growth. This study has documented the ability of rofecoxib to reduce solid tumor growth when administered as a

single agent to Swiss mice whose solid tumors were induced with EAC cells.

Specific inhibition of COX-II in a murine Lewis lung carcinoma model restores host antitumor reactivity by decreasing the immune suppressor cytokine interleukin 10 and increasing the antitumor cytokine interleukin 12[51]. Given the potential for inhibition of COX-II in tumor, stromal, and immune cells, it is not surprising that combination therapy of COX-II inhibitors with antiproliferative agents and radiation therapy result in synergistic benefits in tumor regression[52,53]. Parallel to these results, we have reported the beneficial effect of combination therapy of the COX-II inhibitor, rofecoxib, with cisplatin.

In addition to thalidomide and rofecoxib, the ACE-inhibitor, captopril, was investigated in the present study. A previous study documented the antitumor activity of captopril in rats[28]. On 1999, Prontera *et al.* have also reported that the inhibition of gelatinase A (MMP-2) by captopril was able to reduce tumor growth and lung metastasis in mice bearing Lewis lung carcinoma[37]. In the current study, we documented the ability of captopril to reduce solid tumor growth. This antitumor effect tended to increase when captopril was combined with cisplatin.

Increased life span is a parameter that reflects the efficacy of antineoplastics. Because the majority of cancer treatments involve reducing tumor volume, prolonging life span, and improving quality of life, the ability of treatment regimens to increase life span is important[54]. Thalidomide treatment and its combination with cisplatin produced the highest %ILS, 57.2% and 79.8%, respectively. This may imply the efficacy of thalidomide as an adjuvant treatment to cisplatin. Similar observation was observed with both rofecoxib and captopril.

The antiangiogenic activity of thalidomide, rofecoxib and captopril has been postulated as a possible mechanism of their antitumor effect. To measure angiogenesis, very few methods are available at present. Individual methods rely on the estimation of the degree of neovascularization around the tumor. An example of these assays includes chick embryo and cornea assay[55,56]. From a therapeutic standpoint, these assays may potentially overestimate the activity of certain agents because local drug concentration is higher than drug levels in any other tissue. As capillaries grow toward the stimulus, the drug concentration becomes higher[40]. The present study suggests that the use of the dye-perfusion technique[40] provides an objective endpoint and diminishes the variations. Moreover, this assay was less labor-intensive and required a relatively small number of animals.

Thalidomide was reported to be an angiogenesis inhibitor[8]; therefore, the exploration of this role in tumor development was a part of the present study. Both thalidomide and its combination proved effective as antiangiogenic treatments. The antiangiogenic effect of thalidomide was reported when administered by the intraperitoneal route but not by the oral route[8]. This was explained on the basis of the bioavailability of the active form of the drug at the tumor site. It has been noted that thalidomide is partially decomposed by hydrolysis in rat gastrointestinal tract after oral administration[57]. Also, Koch (58) reported that the required dose of thalidomide for producing acute toxicity in mice was almost 2.5 times higher by oral (10g/kg) administration than the intraperitoneal (4g/kg) administration. The current results indicated an antiangiogenic effect of thalidomide after intraperitoneal administration.

In the study conducted by Kotoh *et al.*[8], thalidomide was effective in ES63 tumor strain only but not in ES80 after intraperitoneal administration. This may indicate that the effect of thalidomide is tumor-type dependent. Until now, the strong antiangiogenic effect of thalidomide has been exclusively noticed in the bFGF- or VEGF-induced corneal angiogenesis model[10]. This indicates that this drug modulates the function of these angiogenic growth factors for inhibition of new vessel formation[8].

A significant reduction in tumor-induced angiogenesis was associated with rofecoxib administration in EAC-bearing mice. The effectiveness of rofecoxib in the inhibition of angiogenesis has been established in this mouse model. The present results indicated that rofecoxib has a significant effect on the inhibition of tumor blood supply.

The ability of captopril to inhibit new blood vessel formation has been demonstrated in capillary endothelial cells[28]. In contrast to thalidomide and rofecoxib, the mild reduction in angiogenesis observed in the present study with captopril was not significant. Similarly, cisplatin produced a marginal reduction in angiogenesis. However, the combination of captopril and cisplatin exhibited an additive depressing effect on angiogenesis that was significantly different from the control. The effect of captopril on angiogenesis is controversial. Other investigators have demonstrated a clear inhibitory effect of captopril on new blood vessel formation[37]. These authors have suggested an inhibitory effect of MMP-2 as a possible mechanism of action for captopril.

It is concluded that thalidomide, rofecoxib, or captopril exerts an antitumorigenic effect in the present model of solid tumor in Swiss mice. Thalidomide and rofecoxib proved to have an antiangiogenic effect in EAC-model. The antiangiogenic effect was associated with a significant reduction in tumor growth and an increase in the life span of animals. Therefore, the present study may suggest a beneficial role of these agents as an adjuvant therapy in tumor management.

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التأثيرات المضادة للأورام والتجدد التكويني للأوعية الدموية لكل من الثاليدومايد، والروفيكوكسيب، والكابتوبريل في خلايا سرطان آرليخ في فئران التجارب البيضاء

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تم القيام بهذه الدراسة لتقييم تأثير كل من الثاليدومايد والروفيكوكسيب والكابتوبريل في نمو الورم السرطاني وإطالة عمر فئران التجارب عند إستخدام كل دواء على حدة أو مجتمعاً مع السيسبلاتين. تم دراسة التأثير المثبط لهذه الأدوية على التجدد التكويني للأوعية الدموية كإحدى الآليات المحتملة للتأثير في نمو للأورام.

تم إحداث ورم سرطاني صلب بحقن خلايا سرطان آرليخ تحت الجلد في الساق اليمنى في فئران التجارب البيضاء. وقد إستخدم هذا الورم لتقييم التأثير المضاد للسرطان وللتجدد التكويني للأوعية الدموية لكل من الثاليدومايد (١٠٠ ملجم/كجم، بالحقن في تجويف البطن) ، الروفيكوكسيب (٢٠ ملجم/كجم،بالفم) ، والكابتوبريل (٥٠ ملجم/كجم،بالفم) لكل دواء على حدة ، أو مجتمعاً مع السيسبلاتين (٢ ملجم/كجم،بالحقن في تجويف البطن). بدأ العلاج بعد حقن الخلايا السرطانية بأربعة وعشرين ساعة. تم قياس حجم الورم كل يومين على مدى إحدى وعشرين يوم. تم متابعة الفئران وتسجيل عدد الفئران الميتة يومياً على مدار مائة يوم. وفي تجربة متزامنة تم حساب درجة التجدد التكويني للأوعية الدموية عن طريق حساب الحجم الوعائي للورم بإستخدام التحليل الطيفي.

وقد وجد أن العلاج بكل من الثاليدومايد ، الروفيكوكسيب ، أوالكابتوبريل على حدة قد نجح في إنقاص حجم الورم ، وأن كل من العلاجات قد زاد من فاعلية دواء السيسبلاتين في إنقاص حجم الورم. كذلك نجح كل من السيسبلاتين و الثاليدومايد على حدة أو مجتمعين في إطالة عمر الفئران لمدة ٨٦ ، ٩٦ ، و١٠٠ يوم على التوالي. نجح كل من الثاليدومايد والروفيكوكسيب في الحد من التجدد التكويني للأوعية الدموية كما أن الجمع بين كل منهما مع السيسبلاتين زاد منهذه الفاعلية. وعلى النقيض لم يثبت أى تأثير مثبط للكابتوبريل على التجدد التكويني للأوعية الدموية عند إستخدامه بمفرده ، على حين أن الجمع بينه وبين السيسبلاتين كان له تأثير مثبط.

تشير نتائج البحث إلى أن الثاليدومايد والروفيكوكسيب والكابتوبريل لهم تأثير مضاد للأورام الصلبة الناتجة عن خلايا سرطان آرليخ في فئران التجارب البيضاء. وقد ثبتت فاعلية الثاليدومايد و الروفيكوكسيب في الحد من التجدد التكويني للأوعية الدموية في هذا النموذج التجريبي للفئران. نستخلص من هذه الدراسة أن الأدوية المثبطة للتجدد التكويني للأوعية الدموية يمكن أن يكون لها دور فعال في زيادة كفاءة العلاج الكيميائي للأورام الصلبة.