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Original Article

Effects of Two-by-Two Combination Therapy with Valproic Acid, Lithium Chloride, and Celecoxib on the Angiogenesis of the Chicken Chorioallantoic Membrane

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Abstract

Background: The synergistic effects of valproic acid (VPA), lithium (Li), and celecoxib (CX) have been shown in combination therapy against the proliferation and metastasis of numerous cancers. Angiogenesis plays a critical role in the pathogenesis of tumor growth and metastasis. The aim of the present study was to evaluate the antiangiogenic effects of VPA, lithium chloride (LiCl), and CX, alone or in 2-by-2 combinations, using the *chicken* chorioallantoic membrane (CAM) assay.

Methods: Fertilized chicken eggs were randomly divided into 10 groups: control, VPA (1.8 and 3.6 µmol/CAM), Li (0.15 and 0.60 µmol/CAM), CX (0.02 and 0.08 µmol/CAM), VPA+Li, VPA+CX, and CX+Li (n=10 per group). A window was made on the eggshells and the CAMs were exposed to a filter disk containing VPA, LiCl, and CX, alone or in 2-by-2 combinations. The control CAMs were treated with distilled water (vehicle). Three days after the treatment, the number of vessel branch points was counted in each CAM. The data were analyzed using SPSS, version 15.One-way ANOVA, followed by the Tukey tests, was used to compare the groups. A $P<0.05$ was considered a statistically significant difference between the groups.

Results: According to the results, all the tested drugs decreased the number of the vessel branch points in a dose-dependent manner compared to the control group ($P<0.001$). In addition, combinations of the drugs were more effective in decreasing angiogenesis than the use of each drug alone.

Conclusion: These findings suggest that 2-by-2 combinations of VPA, CX, and LiCl can be considered an effective antiangiogenesis therapeutic modality.

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Keywords ● Angiogenesis inhibitors ● Combined modality therapy ● Valproic acid ● Celecoxib ● Lithium chloride

What's Known

- Angiogenesis is involved in several pathological conditions, including growth and metastasis of soft tumors.
- Inhibitory effects of valproic acid and celecoxib against the expression of angiogenic factors have been shown previously.

What's New

- Valproic acid, lithium chloride, and celecoxib inhibited the chicken chorioallantoic membrane angiogenesis.
- It seems that combinations of the drugs were more effective in decreasing angiogenesis than the use of each drug alone.

Introduction

Angiogenesis is a physiological process whereby new blood vessels sprout from the preexisting ones. This complex process is controlled via an appropriate balance between angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and angiogenin[1] and antiangiogenic

factors such as thrombospondin-1, endostatin, and angiostatin.[2] Angiogenesis is a necessary step in several physiological conditions, including embryogenesis,[3,4] wound healing,[5] and reconstruction of the uterine endometrium the during menstrual cycle.[6] Nonetheless, unregulated angiogenesis is involved in the pathogenesis of several diseases, including tumor growth and metastasis.[7] Antiangiogenesis therapy has, thus, emerged as a new therapeutic opportunity for the treatment of cancer. In this context, several antiangiogenesis drugs such as monoclonal antibody against human VEGF (bevacizumab; Avastin)[8] and tyrosine kinase inhibitors (sunitinib and erlotinib)[9,10] have been introduced.Nevertheless, not only have most of these drugs demonstrated several side effects[11] but also they have shown limited benefits when used in monotherapy regimens owing to the heterogenic entities of tumors[12] and the induction of drug resistance.[13] Combination therapy, in which 2 or more drugs with different mechanisms are used concurrently for the treatment of a disease, has attracted great attention in this context because it both decreases the toxic side effects arising from the use of high doses of a drug and increases the efficiency of the treatment due to the additive effects that occur between the drugs via different mechanisms.[14]

Valproic acid (VPA), lithium (Li), and celecoxib (CX) are among antiproliferative and antimetastatic drugs used in several combination therapies against numerous cancers.[15-17] The major concern in this context, especially for Li, is the well-known adverse effects of these drugs on several organs, including the kidney, thyroid, and parathyroid. It has, however, been demonstrated that minimizing the dose of Li can ameliorate such adverse effects for patients.[18] Targeting different molecular pathways that are commonly involved in carcinogenesis, low cost, and ease of use make them good candidates for combination therapy. VPA-mediated tumor suppression is mostly exerted through two mechanisms including inhibition of histone deacetylase (HDAC) and regulation of Notch signaling,[19] while the antitumor effects of Li and CX are believed to be mediated through the inhibition of glycogen synthase kinase3β (GSK3β)[15] and cyclooxygenase-2 (COX-2),[16] respectively. In addition to antiproliferative impacts, the antiangiogenic effects of these drugs have been indicated in some previous studies. For example, the association between an increased expression of COX-2 and tumor angiogenesis[20] and the antiangiogenic effects of COX-2 inhibition by CX, mediated through the inhibition of VEGF expression, has been previously demonstrated.[21,22] In the case of Li, in a recent study, Maeng et al.[23] reported the anti-lymphangiogenic effects of Li via the inhibition of GSK3β. The antiangiogenic effects of HDAC inhibition by VPA or other HDAC inhibitors, mediated through the inhibition of VEGF expression, have also been demonstrated in several studies.[24]

Although the synergistic effects of CX, Li, and VPA in tumor cell proliferation and metastasis have been investigated in previous studies, to the best of our knowledge, the possible synergistic effects of CX, Li, and VPA on angiogenesis have yet to be fully elucidated. To that end, in the current study, we treated chorioallantoic membranes (CAMs) with different concentrations of CX, Li, and VPA, alone or in 2-by-2 combinations, and counted the number of vessel branch points (as an index of angiogenesis). The CAM assay is a valid and reliable method commonly used for the evaluation of angiogenesis.[25,26] The aim of the present preliminary study was to evaluate the antiangiogenic effects of VPA, lithium chloride (LiCl), and CX, alone or in combinations, using the CAM assay.

Materials and Methods

Control and Experimental Groups

Fertilized chicken eggs (purchased from the Department of Poultry, School *of* Veterinary Medicine, Shiraz University) on day 10 of embryo development were incubated at 37 °C and 50% humidity with automatic rotation in an incubation system. The eggs were randomly divided into a control group and 9 experimental groups (n=10 per group). The experimental groups were comprised of the VPA-treated groups (1.8 and 3.6 µmol/CAM), LiCl-treated groups (0.15 and 0.60 µmol/CAM), CX-treated groups (0.02 and 0.08 µmol/CAM), VPA+LiCl-treated group (3.6+0.60 µmol/CAM, respectively), VPA+CX-treated group (3.6+0.08 µmol/CAM, respectively), and CX+LiCl-treated group (0.08+0.60 µmol/CAM, respectively). Stock solutions of VPA (120 and 240 mM) and LiCl (10 and 40 mM) were prepared in sterile distilled water. The CX stock solution (1.34 and 5.34 mM) was prepared in dimethyl sulfoxide (DMSO) 0.1%. The control group was treated with a vehicle, including sterile distilled water or DMSO 0.1%, which had no significant effects on angiogenesis. Next, 15 µL of the stock solution or the vehicle was loaded on sterile methylcellulose discs (1 cm^2) and the dried discs were then applied on the CAMs. All the chemicals and drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA).

CAM Assay

On day 10 of embryo development, a 1.0 cm^2 square window was made in the eggshells under sterile condition and the sterile methylcellulose discs, which were loaded with the stock solution or the vehicle, were applied on the CAMs. The drug-treated eggs were incubated at 37 °C and 50% humidity for 72 hours. On day 13 of embryo development, the CAM tissues were removed under filter discs, rinsed in phosphate-buffered saline, and photographed (150X) using a stereomicroscope (Leica Zoom 2000).

Digital Image Analysis

CAM angiogenesis was microscopically evaluated through the quantification of the total number of the vessel branch points using computer-assisted image analysis software (Wimasis Image Analysis). Moreover, the total number of the vessel branch points of a selected area (area under the disc=1 cm^2) was counted (figure 1A). An example of the measurement of the total number of the vessel branch points with digital image analysis is depicted in figure 1B. The validity and reliability of the digital image analysis were tested by comparing the results with those obtained from manual counting of the total number of vessel branch points by a person, who was blinded to the treatments.

Statistical Analysis

The effects of the drugs on CAM angiogenesis were expressed as the total number of the vessel branch points. The data were analyzed using SPSS software (version 16). The Kolmogorov–Smirnov test was used to check the normal distribution of the data. Because the data from the total number of the vessel branch points were normally distributed (P=0.2), one-way ANOVA, followed by the Tukey post hoc test, was employed to compare the mean of the total number of the vessel branch points between the different groups. All the results were reported as mean±SD of at least 10 CAMs. A P<0.05 was considered a statistically significant difference between the groups.

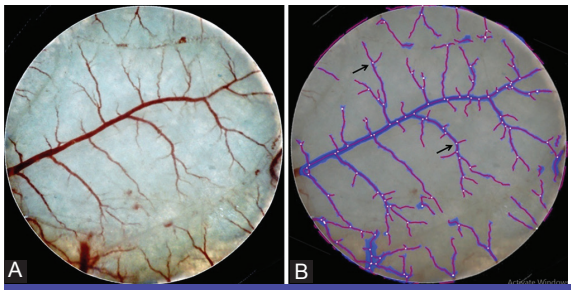

Figure 1: Micrograph (A) shows the distribution of blood vessels in a vehicle-treated chicken chorioallantoic membrane (CAM) in the control group and when analyzed using Wimasis Image Analysis software (B) to quantify the vessel branch points. Lines marked in red are vessels and points marked in white are vessel branch points (arrows).

Results

Effects of VPA on CAM angiogenesis

The CAMs were exposed to VPA-containing disks (1.8 and 3.6 µmol/CAM) to evaluate the effects of VPA on CAM angiogenesis by counting the number of the vessel branch points, as an index of angiogenesis. As can be seen in figure 2B and figure C, the vessels were irregular and brittle. In addition, a significant decrease in the number of the vessel branch points compared to the control CAMs (figure 2A) (P<0.001) was observed upon the treatment of the CAMs with VPA in a dose-dependent manner (figure 2D).

Effects of LiCl on CAM Angiogenesis

LiCl-containing disks (0.15 and 0.60 µmol/CAM) were applied on the CAMs, and the number of the vessel branch points was counted. As is

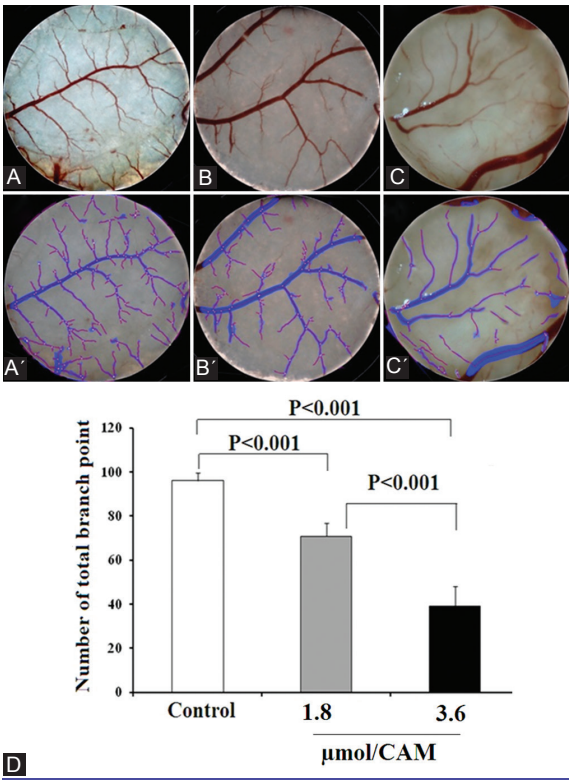

Figure 2: Micrographs show vessel branch points in the control vehicle-treated chicken chorioallantoic membrane (CAM) (A) and analyzed micrograph (A´), CAM treated with 1.8 µmol of valproic acid (VPA) (B) and analyzed micrograph (B´), and CAM treated with 3.6 µmol of VPA (C) and analyzed micrograph (C´). The number of the vessel branch points was counted in each group and analyzed using one-way ANOVA, followed by the Tukey post hoc test (D). The data are presented as mean±SD of the number of the vessel branch points in each group (n=10).

demonstrated in the micrographs of figure 3, the number of the vessel branch points was significantly lowered in the LiCl-treated CAMs compared to the control CAMs. Comparison of the mean vessel number between the control and LiCl-treated CAMs (one-way ANOVA, followed by the Tukey post hoc test) revealed a significant dose-dependent decrease in the number of the vessel branch points in the LiCl-treated group (P<0.001).

Effects of CX on CAM Angiogenesis

The CAMs in the CX group were exposed to CX-containing disks (0.02 and 0.08 µmol/CAM) for 3 days, and the number of the vessel branch points was counted. The micrographs in figure 4 demonstrate the number of the vessel branch points in the control and CX-treated groups. CX treatment lowered the number of the vessel branch points in a dose-dependent manner by comparison with the control CAMs (P<0.001) (one-way ANOVA, followed by the Tukey post hoc test).

Synergistic Effects of VPA, LiCl, and CX on CAM Angiogenesis

The CAMs were treated with 2-by-2 combinations of the drugs: CX (0.08 µmol/CAM)+VPA (3.6 µmol/CAM), CX (0.08 µmol/CAM)+LiCl (0.60 µmol/CAM), and LiCl (0.60 µmol/CAM)+VPA (3.6 µmol/CAM) for 3 days. Thereafter, the number of the vessel branch points was counted. The micrographs in figure 5 demonstrate the number of the vessel branch points in the CAMs treated with the combinations of the drugs. Our findings showed that combination therapy decreased the number of the vessel branch points in comparison to the control CAMs (P<0.001) and monotherapy with each of the aforementioned drugs alone (P<0.001) (one-way ANOVA, followed by the Tukey post hoc test).

Discussion

In the present study, we applied a widely used in vivo angiogenesis model, CAM assay,[25] to

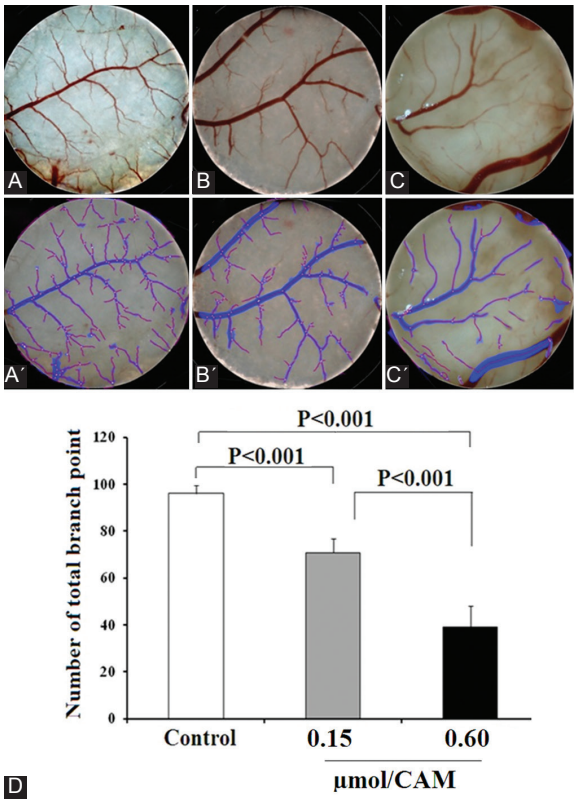

Figure 3: Micrographs show vessel branch points in the control vehicle-treated chicken chorioallantoic membrane (CAM)(A) and analyzed micrograph (A´), CAM treated with 0.15 µmol of lithium chloride (LiCl) (B) and analyzed micrograph (B´), and CAM treated with 0.60 µmol of LiCl (C) and analyzed micrograph (C´). The number of the vessel branch points was counted in each group and analyzed using one-way ANOVA, followed by the Tukey post hoc test (D). The data are presented as mean±SD of the number of the vessel branch points in each group (n=10).

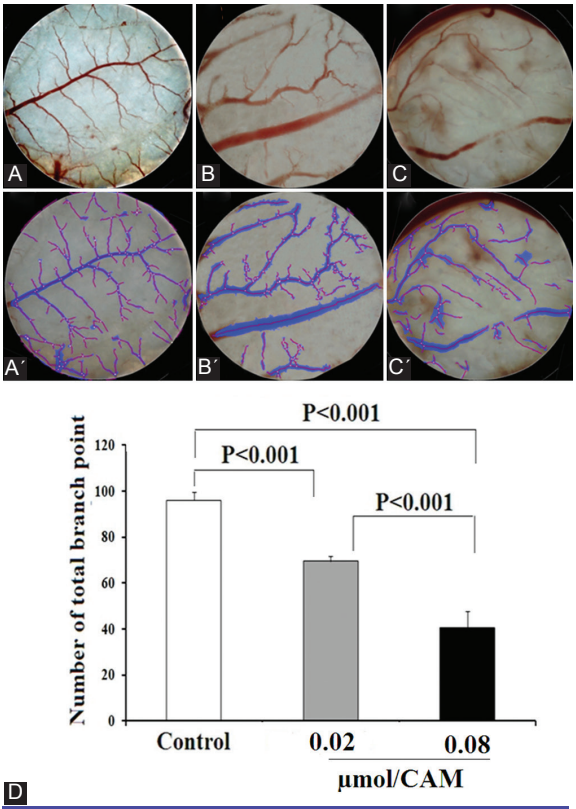

Figure 4: Micrographs show vessel branch points in the control vehicle-treated chicken chorioallantoic membrane (CAM) (A) and analyzed micrograph (A´), CAM treated with 0.02 µmol of celecoxib (CX)(B) and analyzed micrograph (B´), and CAM treated with 0.08 µmol of CX (C) and analyzed micrograph (C´). The number of the vessel branch points was counted in each group and analyzed using one-way ANOVA, followed by the Tukey post hoc test (D). The data are presented as mean±SD of the number of the vessel branch points in each group (n=10).

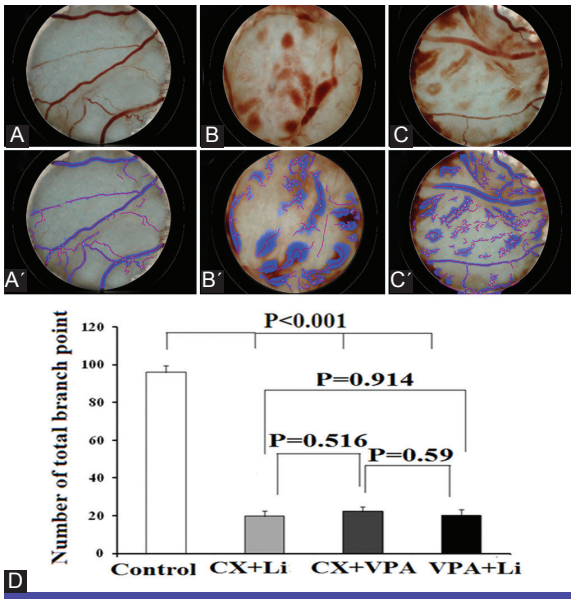

Figure 5: Micrographs show vessel branch points in the Li (0.60 µmol)+VPA (3.6 µmol)-treated chicken chorioallantoic membrane (CAM)(A) and analyzed micrograph (A´), CAM treated with Li (0.60 µmol)+CX (0.08 µmol) (B) and analyzed micrograph (B´), and CAM treated with VPA (3.6 µmol)+CX(0.08 µmol) (C) and analyzed micrograph (C´). The number of the vessel branch points was counted in each group and analyzed using one-way ANOVA, followed by the Tukey post hoc test (D). The data are presented as mean±SD of the number of the vessel branch points in each group (n=10).

evaluate the effects of VPA, Li, and CX alone or in 2-by-2 combinations on angiogenesis. Our findings showed that all 3 drugs reduced the number of vessel branch points as a marker of angiogenesis. When using the 2-by-2 combinations of the drugs, we observed more efficient antiangiogenic effects, which suggested possible additive effects between the antiangiogenic effects of VPA, Li, and CX. Given the role of angiogenesis in the pathogenesis of several diseases such as rheumatoid arthritis, atherosclerosis, diabetic nephropathy and retinopathy, endometriosis, and especially tumor growth and metastasis[27] and on the other hand the side effects of the current antiangiogenesis therapy including anti-VEGF antibodies and tyrosine kinase inhibitors,[11] a combinational therapeutic regimen using VPA, Li, and CX can be considered for the prevention and treatment of such diseases.

Because cancer development is a complex multistep process initiated with cell proliferation and extended with angiogenesis and metastasis, it is vitally important that an anticancer agent capable of targeting all these aspects of carcinogenesis simultaneously be identified. The antiproliferative and antimetastatic impacts of COX-2, HDAC, and GSK3 inhibitors have been clearly demonstrated in a large number of studies conducted on numerous kinds of tumors. Furthermore, these inhibitors exhibit synergistic effects when used concurrently for the treatment of tumors. For instance, it has been demonstrated that combined treatment with VPA and CX induces more apoptosis in neuroblastoma cells than individual drug treatment.[28] Moreover, combination therapy with LiCl and VPA decreases growth via apoptosis in medullary thyroid cancer cells[29] and carcinoid tumor cells[30] in vitro.

Besides its anti-inflammatory effects, CX is reported to exhibit antiproliferative and antimetastatic effects in many tumor cells.[22] In the present study, we found significant and dose-dependent effects of CX at low concentrations in the inhibition of angiogenesis in the CAM assay. Decrease in the expression of angiogenic factors such as angiopoietin-2, VEGF, and bFGF may be a description for the antiangiogenic effects of CX.[31,32] The antiangiogenic effects of CX are also demonstrated in combination therapy. For instance, combined inhibitory effects of CX and curcumin have been reported on the angiogenesis of the CAM.[33] El-sayed et al.[17] recently revealed the anti-inflammatory and antiangiogenic effects of CX with evening primrose oil co-treatment in complete Freund's adjuvant-induced arthritis in rats. Furthermore, a combination of CX and octreotide was found to reduce intrahepatic and splanchnic angiogenesis via downregulation in hypoxia-inducible factor-1α-VEGF signaling pathways.[16]

Historically, VPA and Li have been known as effective drugs for the treatment of neurological disorders such as bipolar disorder.[27] The synergistic effects of VPA and Li, mediated through the inhibition of GSK3 activity, have also been demonstrated for the treatment of drug-resistant bipolar patients and in glutamate-induced neurotoxicity.[34] Aside from neurological effects, the role of GSK3 and HDAC has been explored in the pathogenesis of cancer, which is why the use of VPA and Li for the treatment and prevention of cancers has been considered.[19,35] In the present study, we evaluated the effects of Li and VPA alone or in combination with each other or with CX on CAM angiogenesis. Based on our finding, Li and VPA suppressed CAM angiogenesis in a dose-dependent manner. The antiangiogenesis activity of VPA, observed in our study, is consistent with a previous study reported by Michaelis et al.,[36] who used a similar CAM assay model and demonstrated that VPA inhibited angiogenesis. The inhibitory effects of VPA on vessel formation have also been shown using other angiogenesis models, including the zebrafish model,[37] in vitro Matrigel plug assay,

and mouse model.[26]

In the current study, we observed maximum inhibitory effects on angiogenesis when using combinations of the drugs. The exact molecular mechanisms underlying the antiangiogenic effects of Li and VPA and their synergistic effects are not clear. Even so, downregulation in angiogenesis factors, including VEGF, VEGF receptor, and FGF, by VPA and Li may be an explanation for their antiangiogenic properties.[24,26] As was discussed previously, the synergism between the neuroprotective effects of VPA and Li has been attributed to their inhibitory impacts on GSK activity.[34] Whether or not such a mechanism is involved in the synergistic effects of VPA with Li in the present study remains to be elucidated in future studies.

The present study has 2 major limitations. At first glance, our data demonstrated that the combination of the drugs exerted greater, if not comparable, effects than the sum of the individual effects, suggesting the superiority of combination therapy to monotherapy. Nevertheless, further pharmacological evaluations such isobologram analysis[38] are needed to determine the exact interaction of the drugs. In addition, in the CAM assay, we measured the toxicity of the drugs by evaluating the effects of the drugs on the viability of the embryos.[39] All the embryos in our drug-treated groups were alive during the study period, suggesting that the concentrations of the drugs used in our study (both in monotherapy or combination therapy) had no toxic effects. Nonetheless, more in vivo studies are required to determine and compare the side effects of the drugs in combination or in monotherapy.

Conclusion

Our findings showed that a combined low dose of CX, Li, and VPA synergistically inhibited angiogenesis in the CAM. Therefore, 2-by-2 combinations of these drugs may have beneficial implications for the treatment of cancer and other pathological conditions closely related to angiogenesis. Our findings are, however, preliminary and further studies drawing upon other in vitro and in vivo techniques of angiogenesis are needed to confirm our data and to understand the mechanisms of the synergistic effects of the drugs.

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Conflict of Interest: None declared.

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