

## Chloroquine Used in Combination with Chemotherapy Synergistically Suppresses Growth and Angiogenesis *In Vitro* and *In Vivo*

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**Abstract.** *Background:* The inhibition of autophagy using pharmacological inhibitors such as chloroquine may be an effective strategy to overcome chemotherapy or resistance to anti-angiogenic therapy. *Materials and Methods:* The cytotoxic effect of doxorubicin (0.1-1  $\mu$ M), chloroquine (0.25-32  $\mu$ M) and their combination were investigated by employing ATP assay in human umbilical vein endothelial cells (HUVECs). The effect of doxorubicin and chloroquine combination was also measured using tube formation assay on Matrigel. The anti-angiogenic activities of doxorubicin (2.5  $\mu$ g/pellet) and chloroquine (15  $\mu$ g/pellet), their combination, and standards (50  $\mu$ g/pellet) were tested *in vivo* using the chick embryo chorioallantoic membrane (CAM) assay. *Results:* The combination of doxorubicin and chloroquine significantly had a stronger anti-angiogenic effect than the positive control ( $\pm$ )-thalidomide and doxorubicin alone in the CAM assay and *in vitro* tube-formation assay. *Conclusion:* Chloroquine enhanced the anti-angiogenic effect of doxorubicin on CAM at the tested concentrations.

Doxorubicin, a first-generation anthracycline, is currently one of the most active cytotoxic, chemotherapeutic agents for treating various kinds of cancer such as breast and prostate cancer, childhood solid tumors, leukemia, soft-tissue

sarcoma, aggressive lymphomas and human hepatocellular carcinoma (1-4). It has anticancer activity by altering topoisomerase II activity, as well as inhibiting DNA-dependent DNA and RNA synthesis through multiple mechanisms including intercalation with DNA strands and generation of reactive oxygen species (ROS) (4, 5). This topoisomerase II-mediated DNA damage is followed by growth arrest in G<sub>1</sub> and G<sub>2</sub> and programmed cell death (3).

Despite its highly potent anticancer activity against various tumor cell types, doxorubicin has a low therapeutic index and, due to its associated acute and chronic toxicities including myelosuppression, immunosuppression, and cardio- and renal toxicity, its clinical use in cancer treatment is limited. Therefore, lowering its toxicity by combining this agent with another highly effective novel non-toxic agent, it is still a high priority need. Besides reducing the side-effects of doxorubicin, a combination treatment might also modulate different signaling pathways in cancer cells (4, 6). In this context, various drugs and molecules, such as the lipid-lowering agent probucol (7), all-*trans* retinoic acid (4), noscapine (6), zoledronic acid (8), coenzyme Q10-liquid crystalline nanoparticles (5), SU5416 (9) and anti-angiogenic agents such as sirolimus (10), tetrathiomolybdate (11), combretastatin A4 (12), in combination with doxorubicin were reported to lead to enhanced antitumor efficacy.

Chloroquine, a well-known anti-malarial drug, is used in the clinic to treat auto-immune diseases such as lupus erythematosus and rheumatoid arthritis by inhibiting the immune system and due to its anti-inflammatory properties; it is also used for retroviral purposes (13, 14). It is known to show potent cytotoxic effects against various cancer cell types such as human cervical, colorectal, lung, breast, and hepatocellular and mouse colon cancer cells (15, 16).

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*Key Words:* Doxorubicin, chloroquine, angiogenesis, CAM assay, tube formation.

The anticancer properties of chloroquine are due to its ability to induce cell-cycle arrest, inhibit autophagy and promote apoptosis in tumor cells (14, 16, 17). As a lysosomotropic agent, chloroquine sensitizes various cancer cells to the antiproliferative effect of radiation, chemotherapy, cisplatin, 5-fluorouracil and oxaliplatin by inhibiting lysosomal acidification (16, 18). Autophagy, a conserved catabolic process that includes degradation of cytoplasmic proteins and organelles for further intracellular recycling, plays a controversial role in tumorigenesis due to its protective or degenerative properties in tumor cells during tumor progression (19). Dysregulation of autophagy may occur in a manner that enables tumor cell survival even in a limited-nutrient environment depending on cancer type (18). Chloroquine has already been reported to restore drug sensitivity and increase the intracellular targets of drugs by inhibiting the degradation of autophagic cargo and by blocking autophagic flux (17). Because of its highly safe profile as compared to other autophagy inhibitors, chloroquine might be more appropriate for use in combinatorial cancer therapy with other chemotherapeutics as a cancer-specific chemosensitizer (15, 16).

Angiogenesis, the formation of new blood vessels from existing ones, plays a vital role in embryo growth, wound healing, post-ischemic tissue restoration and endometrial changes during the menstrual cycle physiologically, but also acts in inflammation, several autoimmune diseases such as lupus erythematosus, and cancer (13, 20). In this light, we aimed to investigate doxorubicin and chloroquine for their anti-angiogenic properties alone and in combination using the chicken chorioallantoic membrane (CAM) assay. The *in vivo* CAM assay is very useful, well-established and an animal alternative method for screening angiogenic, anti-angiogenic and irritation potency of substances as well as therapeutics (21, 22). To the best of our knowledge this is the first study on the angiogenic evaluation of doxorubicin and chloroquine combination using the CAM assay.

## Materials and Methods

**Cell culture and chemicals.** Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in EBM-2 endothelial cell basal medium (Lonza, Walkersville, MD, USA) with full supplements endothelial growth medium-2 (EGM-2) bullet kit : 2% fetal bovine serum (FBS), 0.4% human fibroblast growth factor-2 (hFGF-2), 0.1% vascular endothelial growth factor (VEGF), 0.1% recombinant analog of insulin-like growth factor-1 (R3-IGF-1), 0.1% human epidermal growth factor (hEGF), 0.04% hydrocortisone, 0.1% ascorbic acid, 0.1% heparin, 0.1% gentamicin, amphotericin b (GA-1000) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Chloroquine (Sigma, St. Louis, MO, USA) was dissolved in deionized water at a concentration of 50 mM as a stock solution; reference anti-angiogenics (prednisone, cortisone, and racemic (±)-thalidomide; Sigma-Aldrich Chemie GmbH Munich,

Germany) were dissolved in dimethylsulfoxide (DMSO) as a stock solution (500 mM) and doxorubicin was dissolved in Molecular Biology water at 2 mg/ml concentration as a stock solution. The highest concentration of DMSO did not exceed 0.1% (v/v) in the cell culture. Both doxorubicin (Kocak Farma, Turkey) and chloroquine aliquots were stored at -20°C for further use and any further dilutions were made in culture medium.

**ATP viability assay.** The cytotoxic effects of doxorubicin, chloroquine and their combination on HUVECs were examined with an ATP assay. Briefly, cells were seeded at a density of 5×10<sup>3</sup> cells per well of 96-well plates in triplicate and incubated overnight. After 24-h incubation, cells were pretreated with chloroquine (0.25-32 μM) for 24 h prior to a 48-h treatment with doxorubicin (0.1-1 μM). Cells were also treated with reference anti-angiogenics (cortisone, thalidomide, prednisone) at concentrations of 0.1-400 μM for 48 h. Reference anti-angiogenics were prepared in DMSO (final concentration of dimethyl sulfoxide, 0.1%).

In order to carry out the ATP assay, at the end of the 48-h treatment period, 150 μl of medium was removed from each well and 50 μl of somatic cell ATP-releasing agent (ATP Bioluminescent Somatic Cell Assay Kit; Sigma, Steinheim, Germany) was added. After mixing thoroughly, the microplate was allowed to stand for 30 min at room temperature. At the end of the incubation, 50 μl of mixture from each well was transferred to a white non-translucent plate. To each well of the plate, 50 μl of ATP Assay Mix (ATP Bioluminescent Somatic Cell Assay Kit, Sigma, Steinheim, Germany) was added and the emitted light was measured using a 96-well Microplate Luminometer (Bio-Tek, Winooski, VT, USA). ATP viability assays were performed at least twice and the results are given as mean±SD of independent experiments.

**Tube formation assay.** The effect of doxorubicin and chloroquine combination on morphogenesis of endothelial cells was evaluated using the tube formation assay on Matrigel (BD Biosciences, Road Billerica, MA, USA). Briefly, 50 μl Matrigel was added to each well of pre-chilled 96-well plate and incubated at 37°C for 30 min to polymerize the Matrigel. Cells at a density of 2×10<sup>4</sup> cells/well were seeded on a Matrigel-pre-coated 96-well plate and treated with doxorubicin, chloroquine alone or their combination at different concentrations for 2, 6, 12, 16 h. DMSO and reference anti-angiogenics were included as negative and positive controls, respectively. Tube formation was checked quantitatively under an inverted microscope.

**CAM assay.** Fertilized eggs were obtained from Has Tavuk Company (Bursa and Sivrihisar, Turkey). Agarose was purchased from Invitrogen (Thermo Fisher Scientific Waltham, MA, USA) and sodium dodecyl sulphate (SDS) from Fluka-Biochemika (Fluka-Biochemika, St Louis, MO, USA).

Doxorubicin (0.25 mg/ml), chloroquine (1.5 mg/ml) and standards (5 mg/ml) were dissolved in a 2.5% (w/v) agarose solution. For ease of application, pellets of these solutions (10 μl) were prepared by applying dropwise onto circular stainless-steel supports of 5 mm diameter, cooling to room temperature for solidification and subsequently applying onto CAMs.

The *in vivo* CAM assay was performed as described previously by our research group (23). Fertilized hen eggs previously incubated for 72 h at 36.5°C and at a relative humidity of 80% were positioned in a horizontal position and rotated several times. Albumin (8-10 ml)

Table I. Semi-quantitative scoring system of anti-angiogenic effects on chick embryo chorioallantoic membrane (CAM) after treatment (23, 24).

Scale	Anti-angiogenic effect	Effects observed on CAM after treatment
<0.5	Inactive	No change (normal embryo growth).
0.5-0.75	Weak	No capillary-free area.
>0.75-1	Strong	Area with reduced density of capillaries around the pellet not larger than its own area. Small capillary-free area or area with significantly reduced density of capillaries.
>1	Very strong	Effects not larger than double the size of the pellet. Capillary-free area around the pellet at least double the size of the pellet.

Formula used for scoring: Average score=[Number of eggs (score >1)×2+number of eggs (score >0.75-1)×1]/(total number of eggs scored).

was aspirated from a small hole punched with a injector needle in the bottom of the egg. Then a hole on the top of the egg shell of 2 cm diameter was opened by forceps. The cavity in the egg shell was covered with stretch film and eggs were incubated at 36.5°C at a relative humidity of 80% for another 72 h. Pellets (1 pellet/egg) were placed on the CAM which had approximately a diameter of 2 cm and the egg shell hole again covered with stretch film. Twenty-four hours later, the eggs were evaluated under a stereo-microscope (Leica, Wetzlar, Germany) with Leica Application Suite, Las V4.7 (Leica); 10-15 eggs were utilized for each test condition (controls, doxorubicin or chloroquine alone or their combination). For all conditions, three independent experiments were performed.

Anti-angiogenic effects were evaluated by using a scoring system (see Table I) (23,24). The anti-angiogenic scores were calculated using the following formula: Average score=[Number of eggs (score >1)×2+number of eggs (score >0.75-1)×1]/(total number of eggs scored).

As positive controls prednisone, cortisone, thalidomide and negative control SDS at 50 µg/pellet were also tested. CAMs treated with solidified agarose-solution in pellet form (2.5%, w/v) were also tested as a blank. Tests for each compound were performed in triplicate.

*Statistical analysis.* Statistically analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test with Graphpad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) statistical software for Windows. *p*-Values of 0.05 or less were considered statistically significant. Results are expressed as mean±standard deviation.

## Results

*Anti-growth effect of doxorubicin and chloroquine combination by ATP assay.* The anti-growth effect of doxorubicin and chloroquine combination was investigated against HUVECs by ATP assay. The viability upon chloroquine treatment was determined after treatment with a concentration range of 0.25 to 32 µM for 24 h (Figure 1A). In the same manner, the viability of cells upon doxorubicin treatment was assessed after treatment with different concentrations (0.1-1 µM) of doxorubicin for 48 h (Figure 1B). Results show that the doxorubicin exhibited an anti-growth effect in a dose-dependent manner. On the basis of

these growth curves, the stronger growth-inhibitory doses of doxorubicin (0.4, 0.5, 0.8, 1 µM) were selected and combined with different doses of chloroquine (Figure 2). The results showed significant decrease in cell viability especially at 0.8 µM doxorubicin plus 4 µM chloroquine and 1 µM doxorubicin plus 8 µM chloroquine treatment groups (*p*<0.05 and *p*<0.01, respectively).

Cells were also treated with reference anti-angiogenics (cortisone, thalidomide, and prednisone) at concentrations of 0.1-400 µM for 48 h. Reference anti-angiogenics did not demonstrate any cytotoxic effect on HUVECs after 48-h treatment (Figure 3), except cortisone and prednisone at 400 µM dose. As shown in Figure 4, control HUVECs, plated on Matrigel and incubated with control medium, were able to form capillary-like structures while reference anti-angiogenics used as positive control slightly reduced tube formation in a dose-dependent manner (Figure 4A). Doxorubicin blocked tube formation both at 0.8 and 1 µM doses within 16 h; however, chloroquine had no effect on tube formation at 4 or 8 µM after 16 h (Figure 4B). In contrast, treatment with chloroquine at 4 µM combined with 1 µM doxorubicin, and at 8 µM with 0.8 or 1 µM doxorubicin significantly reduced tube formation after 16 h (Figure 4C).

*Anti-angiogenic effects of doxorubicin, chloroquine and their combination.* The anti-angiogenic activities of doxorubicin, chloroquine and their combination were examined using the CAM assay. Prednisone, cortisone, and thalidomide were used as positive controls, and SDS was employed as negative control for the assay. The results of evaluation of the anti-angiogenic effect are summarized in Table II.

According to the microscopic evaluations (Figure 5), the combination of doxorubicin at 2.5 µg/pellet and chloroquine at 15 µg/pellet showed very strong anti-angiogenic effect with a score better than that of doxorubicin alone. On the contrary, chloroquine alone showed no anti-angiogenic effect compared to the positive controls prednisone, cortisone and thalidomide at 50 µg/pellet. Importantly, doxorubicin and

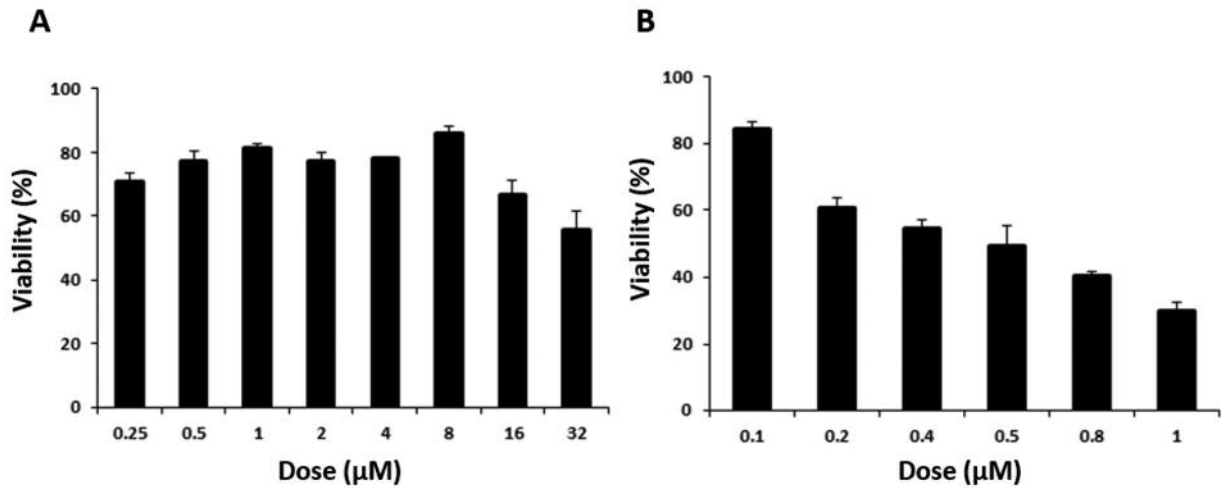


Figure 1. Assessment of viability of human umbilical vein endothelial cells after treatment with different doses of chloroquine for 24 h (A) and doxorubicin for 48 h (B) using the ATP assay. The results are representative of two independent experiments.

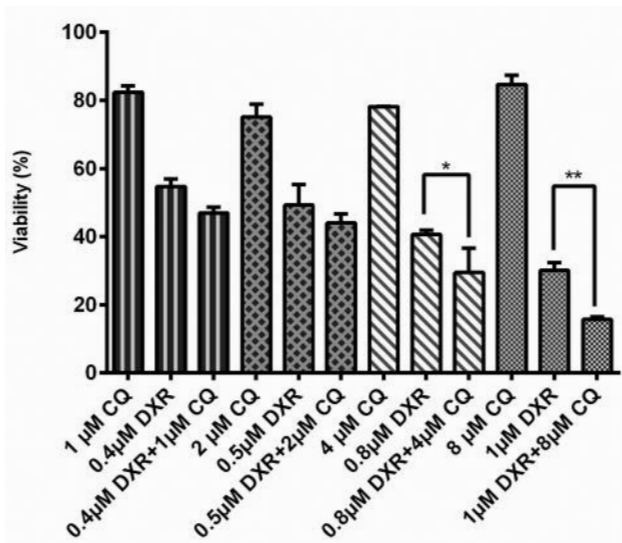


Figure 2. The anti-growth effects of combinatorial treatment with chloroquine (CQ) and doxorubicin (DXR) on human umbilical vein endothelial cells were measured using the ATP assay after 48 h treatment. Statistically significant different at: \* $p < 0.05$  and \*\* $p < 0.01$ .

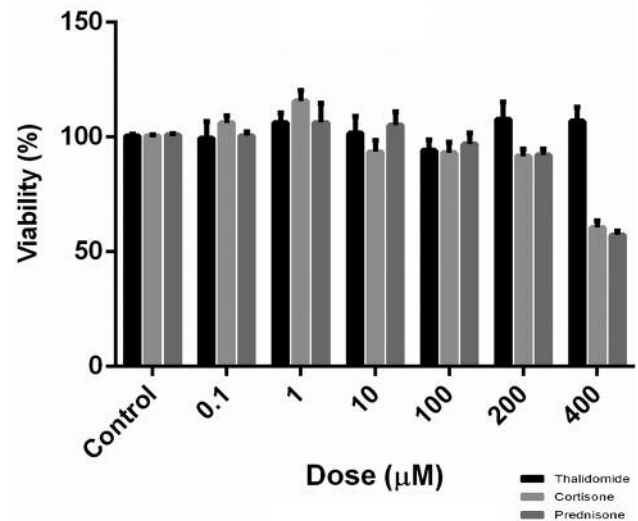


Figure 3. Determination of viability of human umbilical vein endothelial cells after 48 h treatment with reference anti-angiogenics by ATP assay. The data were obtained via two independent experiments.

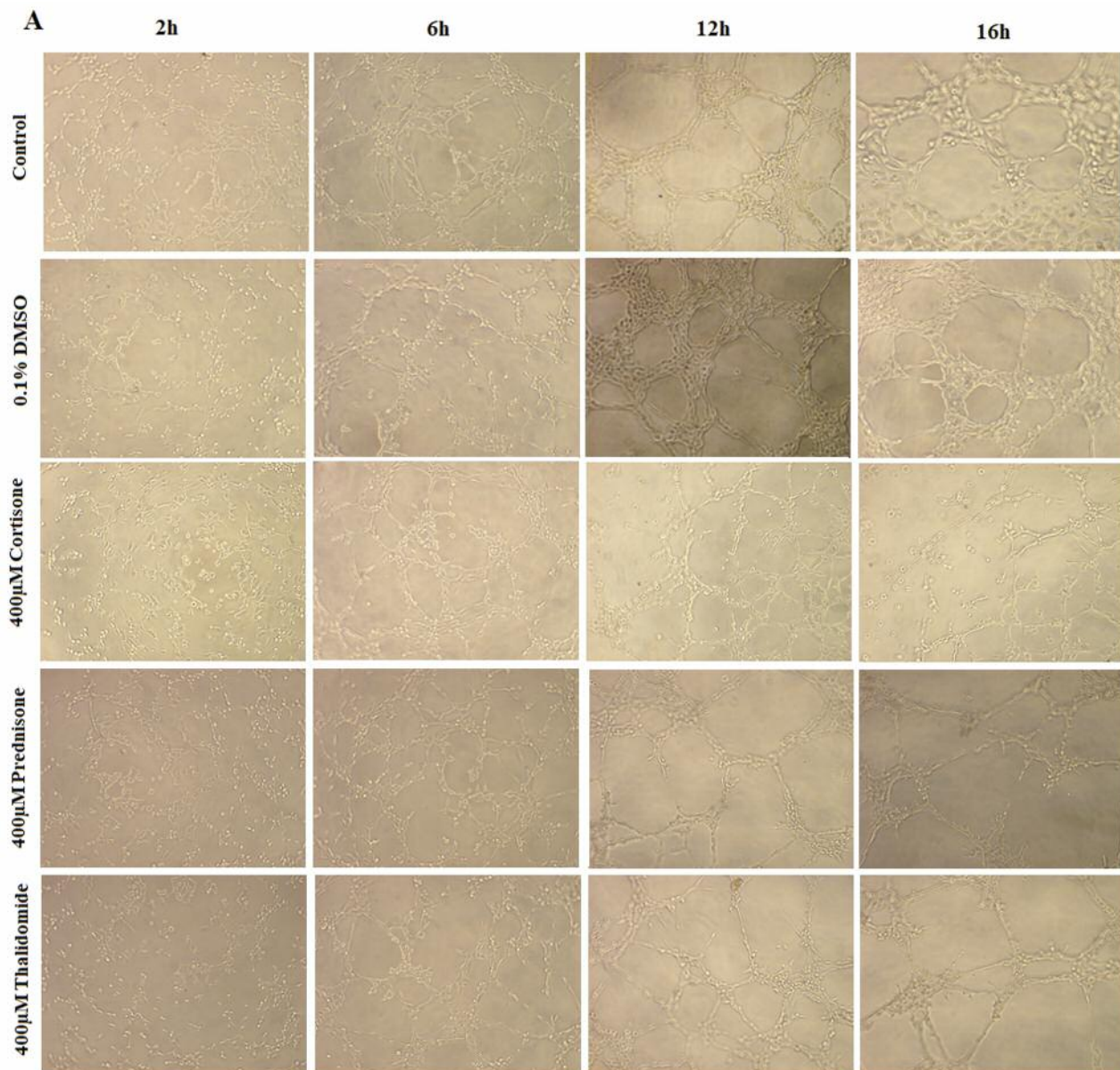
chloroquine combination did not lead to membrane irritation nor toxicity at the tested concentration.

**Discussion**

There has been considerable interest in the potential antitumor effects of therapies combining chemotherapeutic

agents and autophagy inhibitors for the treatment of cancers (25).

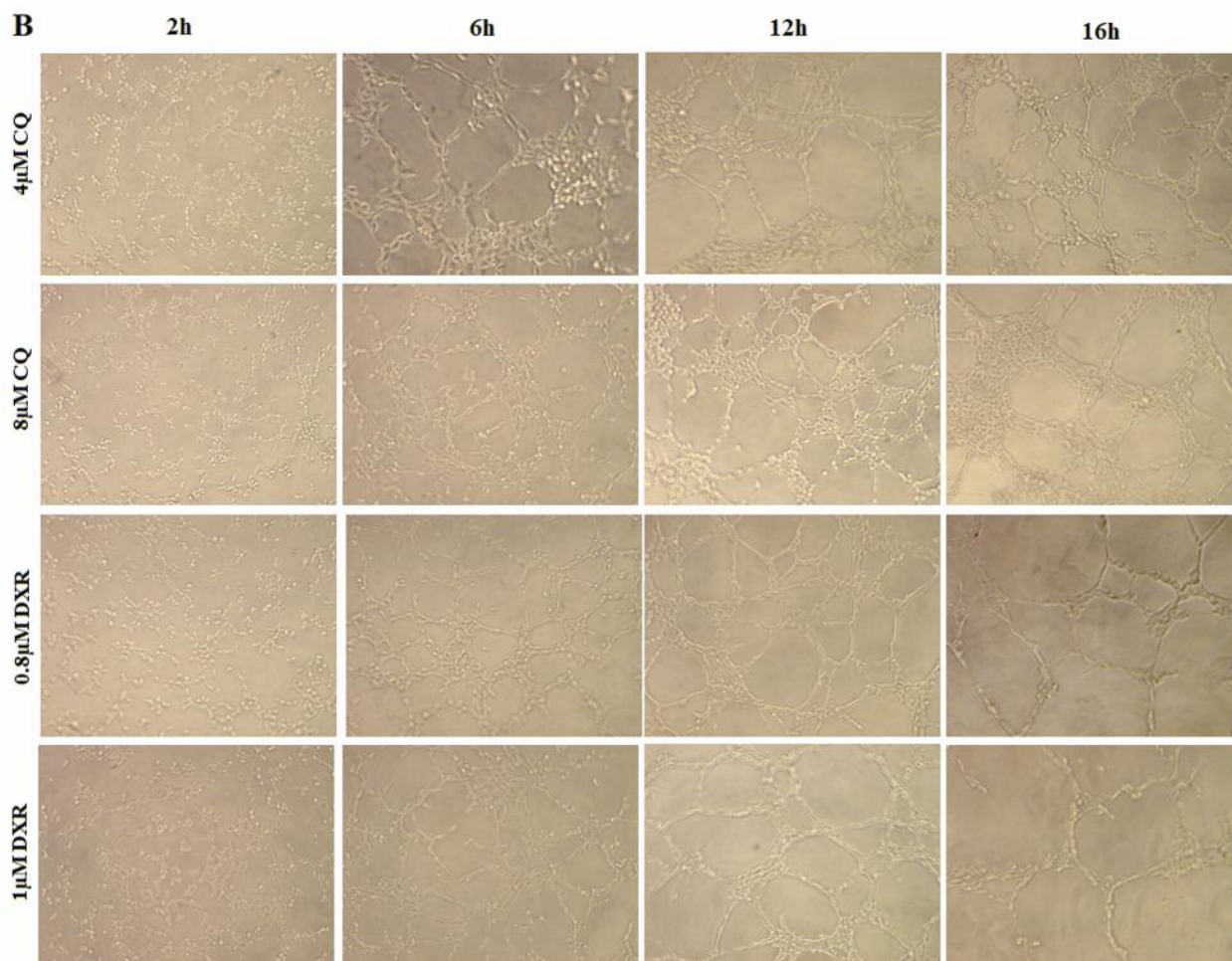
In this study, we showed that doxorubicin in combination with chloroquine exhibited significant anti-growth effect against HUVECs in a dose-dependent manner. Amongst all combinations tested, the combination of doxorubicin at a concentration of 1 μM and chloroquine at a concentration of

Figure 4. *Continued*

8  $\mu\text{M}$  for 48 h was found to be the most effective (Figure 1). The results regarding the concentration of doxorubicin seem to be in good agreement with the clinically relevant concentrations of the drug. Effective doses of doxorubicin used in this study were similar to those used in other studies, which ranged from 0.1  $\mu\text{M}$  to 25  $\mu\text{M}$  for HUVECs (26, 27). More importantly the use of the autophagy inhibitory drug in combination with doxorubicin resulted in slightly better activity compared to doxorubicin alone.

It is well established that HUVECs are organized into lumen-like structures when cultured on Matrigel (23, 28-30). Therefore, we further examined the anti-angiogenic effects of doxorubicin, chloroquine and their combination on tube formation in HUVECs.

HUVECs cultures were treated with different concentrations of chloroquine, doxorubicin or their combination. A previous study demonstrated chloroquine inhibited tube networks of HUVECs (20-40  $\mu\text{M}$ ) and also

Figure 4. *Continued*

restrained angiogenesis in the CAM (10 mg/ml) at higher doses. In this experiment, chloroquine was used at lower doses in both tube formation (4-8  $\mu\text{M}$ ) and CAM assay (1.5 mg/ml) and failed to block formation of tube-like structures even after 16 h of incubation (31). Unlike chloroquine, treatment with doxorubicin resulted in a dose-dependent inhibition of capillary tube formation compared to vehicle control (0.1% DMSO). This was more prominent at 16 h post doxorubicin addition. Moreover, at all combination doses HUVECs formed significantly fewer and thinner tubes compared to the corresponding doses of doxorubicin and chloroquine alone (Figure 4).

Anti-angiogenic therapy aimed at blocking new blood vessel formation in pathologies such as tumour or inflammatory diseases such as rheumatoid arthritis are currently emerging. Angiogenesis associated with tumor growth is believed to promote the proliferation, invasion and

metastasis of cancer cells. Multiple growth factors involved in angiogenesis, including VEGF, have also been demonstrated to play a vital role in cancer progression and metastasis (32). Inhibition of angiogenesis is currently emerging as a novel probably more potent alternative approach to developing cytotoxic agents for cancer treatment, and among these findings, researchers are working on developing new anti-angiogenic strategies (33, 34).

Recent preclinical and clinical evidence has indicated the important role that angiogenesis plays in tumor progression (20, 35, 36). Therefore, inhibition of angiogenesis is thought to be an important strategy in suppressing tumor growth. In order to better resemble the *in vivo* conditions, the CAM model was used to investigate the anti-angiogenic effects of doxorubicin and chloroquine. Data presented herein demonstrate that the two agents can be used in combination to inhibit angiogenesis. Such a combination could clearly be

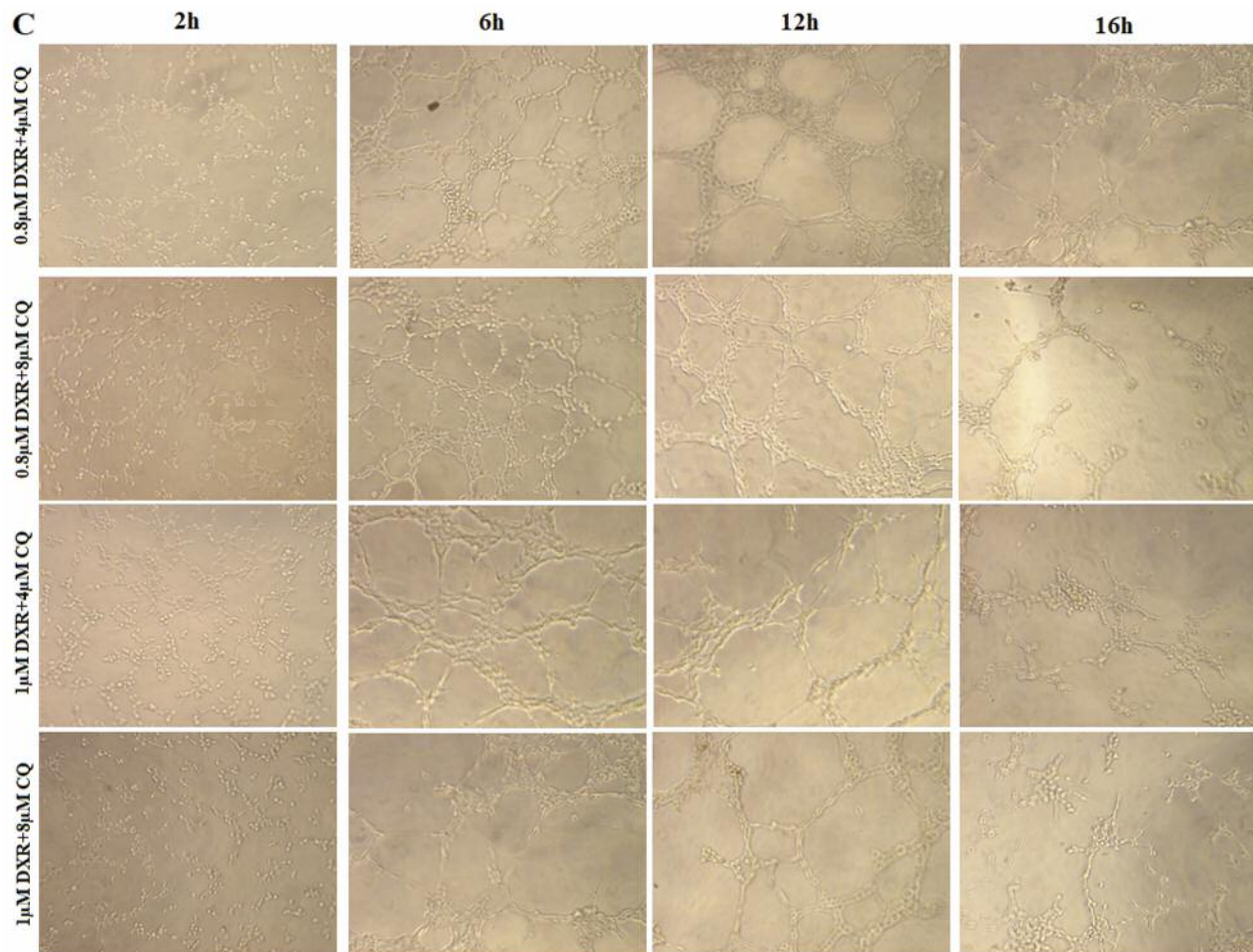


Figure 4. Micrographs showing the effect of reference anti-angiogenics (A), doxorubicin (DXR) and chloroquine (CQ) as single agents (B) and their combination (C) on tube formation by human umbilical vein endothelial cells (HUVECs). The networks of tube-like structures were photographed at 2, 6, 12, 16 h following the addition of HUVECs to Matrigel. In CQ and DXR combinatorial-treated groups, tube formation was reduced compared to both agents alone in a time- and dose-dependant manner.

Table II. Anti-angiogenic effects, positive controls, doxorubicin and chloroquine alone and their combination in the chick embryo chorioallantoic membrane assay.

Sample	Concentration (µg/pellet)	Average score <sup>a</sup>	Anti-angiogenic effect	Irritation (%)
Doxorubicin	2.5	1.2±0.3*	Very strong	17±4
Chloroquine	15	0.4±0.1	Inactive	-
Doxorubicin + chloroquine	2.5+15	1.5±0.6*	Very strong	-
Agar (blank)	2.5%, w/v	0.2±0.2	Inactive	-
Prednisone (positive control)	50	0.9±0.7	Strong	-
Cortisone (positive control)	50	1.2±0.2	Very strong	-
Thalidomide (positive control)	50	0.9±0.2	Strong	-
Sodium dodecyl sulfate (negative control)	50	0.1±0.1	Inactive	87±5

<sup>a</sup>Values are mean average score±standard deviation (n=45 for the test substances and n=30 for standards). Significantly different at \* $p$ <0.05 compared to positive control thalidomide. <sup>b</sup>The irritation (%) means the percentage of the eggs in which irritation is observed.

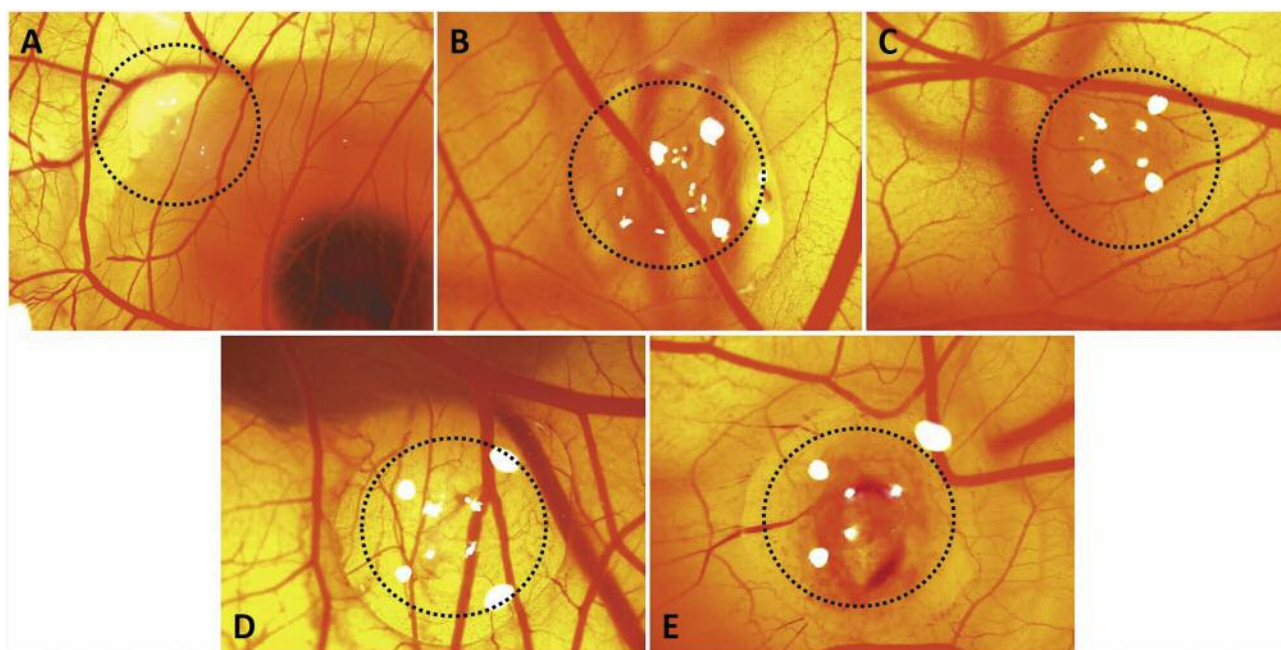


Figure 5. Assay results for chick embryo chorioallantoic membrane (CAM) treated with: A: 50 µg/pellet thalidomide; B: 50 µg/pellet cortisone; C: 2.5 µg/pellet doxorubicin; D: 15 µg/pellet chloroquine; E: 2.5 µg/pellet doxorubicin with 15 µg/pellet chloroquine. While doxorubicin alone had a strong anti-angiogenic, chloroquine was ineffective. The combination of doxorubicin and chloroquine had a very strong anti-angiogenic effect.

advantageous is it may enable the use of lower doses of doxorubicin, which has important side-effects such as cardiotoxicity (a dose limiting toxicity) while improving the efficiency of this important chemotherapeutic (37).

The antitumor anthracyclines doxorubicin, daunorubicin and epirubicin have been reported to inhibit angiogenesis by reducing vascular density and collagenous protein biosynthesis in the CAM. Tube formation by HUVECs on Matrigel has also been shown to be inhibited in the presence of these anthracyclines (38).

Chloroquine has already been reported to enhance anticancer activity in combination therapy with many therapeutic agents such as 5-fluorouracil (19) and lidamycin (39). Our results are in accordance with findings that when chloroquine combined with sunitinib, not only increased apoptotic response, but also cause reduced angiogenic response compared to treatment with each compound alone (16). Importantly, chloroquine enhances delivery to and efficacy of chemotherapeutic agent on the tumor vessel (40). Hence we assumed that chloroquine facilitates overcoming angiogenesis by combination with chemotherapeutic agent.

According to the results for anti-angiogenic activity obtained under the experimental conditions tested herein, we conclude that combination of doxorubicin with chloroquine showed synergistic activity in the CAM assay, at least partially, because of the inhibitory effect of chloroquine on

of autophagy in the CAM. These results also suggest that chloroquine enhances the anti-angiogenic activity of doxorubicin.

Anti-angiogenic therapy and angioprevention, including targeting angiogenesis for the treatment or even for prevention of cancer, inflammation and of such pathologies, looks very promising for the development of new or improved anti-angiogenics (41). To the best of our knowledge, the present study is the first to evaluate the anti-angiogenic of the combination of doxorubicin and chloroquine. The data obtained herein suggest that this combination warrants further thorough investigation, and of course animal experiments for proof-of-concept of a novel promising combination in cancer therapy.

### Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

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