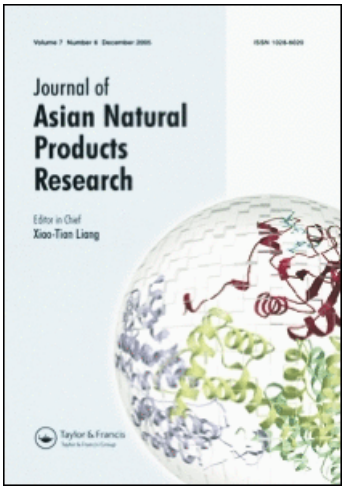

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ANTI-INVASION AND ANTI-ANGIOGENESIS EFFECT OF TAXOL AND CAMPTOTHECIN ON MELANOMA CELLS

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Two highly invasive melanoma cell lines B16BL6 and B16F10 were used to investigate the anti-invasion and anti-angiogenesis action of taxol and camptothecin (CPT). The adhesion of melanoma cells was tested by optical absorbance at 545 nm. The invasive activity of these cells was tested in a transwell cell chamber assay. The cell migration within a 3D collagen matrix was recorded with a time-lapse video recorder and analyzed by computer-assisted cell tracking. Gelatin zymography was used to study the metalloproteinase activity. The chicken chorioallantoic membrane (CAM) model was used to study the anti-angiogenesis effect of the two drugs. The results demonstrated that both taxol and CPT could inhibit the migration of B16F10 cells, and inhibit the adhesion of B16F10 to fibronectin and laminin. They can reduce the metalloproteinase secretion of HT1080 and exhibit the anti-angiogenesis effect in the CAM model. Taxol showed a highly anti-invasion effect on B16BL6 cells while CPT did not exhibit such an effect.

Keywords: Taxol; Camptothecin; Melanoma cells; Adhesion; Invasion; Metalloproteinase

INTRODUCTION

Cancer metastasis is a very complicated process involving many steps and the influence of many factors [1] and is one of the greatest obstacles in clinical treatment. The transition from *in situ* tumor growth to metastatic advanced disease involves the ability of the tumor cells to invade the local tissue and to cross tissue barriers. To initiate the process, carcinoma cells must penetrate the epithelial basement membrane. The adhesion of tumor cells to extracellular matrix (ECM), such as fibronectin, laminin, matrigel, is crucial for metastasis [2–4]. This is followed by ECM degradation which is believed to include secretion and activation of proteolytic enzymes, such as matrix metalloproteinases (MMPs) [5] which degrade extracellular matrix components, such as type IV collagen, glycoprotein and proteoglycans. Proteolytic modifications of the matrix barriers are followed by pseudopodial protrusion and locomotion of tumor cells. Therefore, the mobility of tumor cells is also

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a major factor in cancer metastasis. Angiogenesis, or the growth of new blood vessels from preexisting vessels, is essential for supporting tumor growth and progression not only by providing the necessary blood supply but also by allowing metastatic cells into the circulation [6–8]. It has attracted more attention in recent years and it has been a new target in cancer metastasis.

Taxol [9–14] and camptothecin (CPT) [15–20] and their analogues have been widely used as cytotoxic drugs in cancer chemotherapy. Their potent inhibition effects on cancer metastasis are of great interest; taxol was found to have anti-proliferation, anti-migration effect on human glioma cells and anti-invasion effect on Ovcar-3 cell invasion [21,22]. Topotecan was reported to inhibit VEGF- and bFGF-induced migration of human umbilical vein endothelial cells (HUVEC) [23]. But there are fewer comparative studies of anti-metastatic mechanisms of the two drugs. Liu [24] reported the anti-tumor and anti-invasion activity of taxol, harringtonine, homoharringtonine and CPT on highly metastatic melanoma B16BL6 and HT-1080 cells. In this study, we used two highly invasive melanoma cell lines, B16BL6, B16F10 (descendants of less metastatic B16), and different methods to clarify the characteristics of the anti-invasion and anti-migration effects of taxol and CPT.

Vascular proliferation is a requirement for solid tumor growth [25,26] and blood vessel counts have been found to correlate significantly with metastases and prognosis in breast cancer, non-small cell lung cancer and melanoma [27,28]. Many *in vitro* models have been used to study the anti-angiogenesis effect of taxol, CPT and their analogues [29,30]. In this study, we used the *in vivo* chicken chorioallantoic membrane (CAM) model to compare the anti-angiogenesis effects of taxol and CPT.

RESULTS AND DISCUSSION

Anti-adhesion Effects

The adhesion inhibition of B16F10 to fibronectin by taxol and CPT are shown in Fig. 1. Taxol $10^{-8}\,\mathrm{mol\,l^{-1}}$ (42% ± 2.51) and CPT $10^{-8}\,\mathrm{mol\,l^{-1}}$ (37% ± 5.77) showed higher inhibitory effects than that of taxol $10^{-10}\,\mathrm{mol\,l^{-1}}$ (28% ± 8.66) and CPT $10^{-10}\,\mathrm{mol\,l^{-1}}$ (16% ± 10.25) compared with the control group (100% ± 9.29). The adhesion inhibition of B16F10 to laminin by taxol and CPT are shown in Fig. 2. Inhibition effect of taxol $10^{-8}\,\mathrm{mol\,l^{-1}}$ and $10^{-10}\,\mathrm{mol\,l^{-1}}$ group were 39% ± 3.48 and 32% ± 10.24, that of CPT $10^{-8}\,\mathrm{mol\,l^{-1}}$

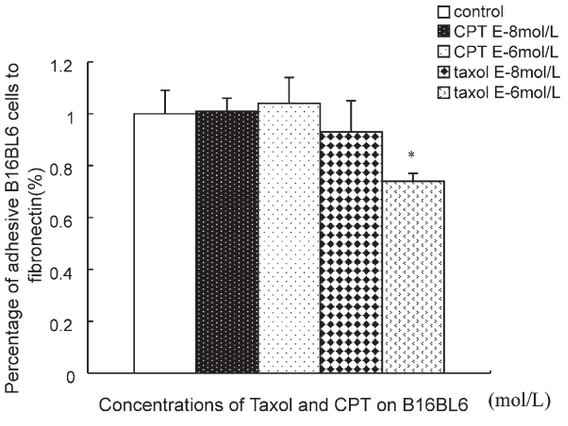

FIGURE 1 The anti-adhesive effect of Taxol and CPT on B16BL6 to fibronectin.

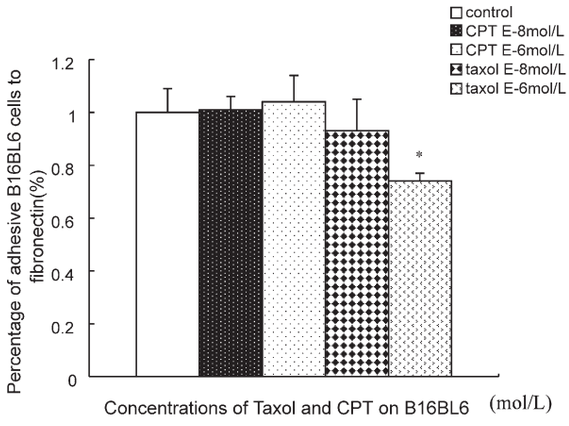

FIGURE 2 The anti-adhesive effect of Taxol and CPT on B16BL6 to laminin.

and $10^{-10}\,\mathrm{mol}\,\mathrm{l}^{-1}$ groups were $13\% \pm 1.53$ and $4\% \pm 5.23$ separately compared with the control group ($100\% \pm 2.9$).

Anti-invasion Effects

The number of the B16BL6 cells in the control group was 135 ± 9.34 per membrane, in the taxol 10^{-9}, 10^{-8}, $10^{-7}\,\mathrm{mol}\,\mathrm{l}^{-1}$ group were 101 ± 9.85, 61 ± 12.32, 31 ± 7.45 per membrane, separately (Fig. 3). The cells treated with taxol showed lower invasive capacity as compared with the control group and the inhibition effect was concentration dependent (Fig. 4). Interestingly CPT did not exhibit an inhibition effect at the same concentration.

Effects on the Collagenase Activity

The secretion feature of type IV collagenases of HT1080 exposed to taxol and CPT is shown in Fig. 5. The data show that taxol ($10^{-6}\,\mathrm{mol}\,\mathrm{l}^{-1}$) and CPT ($10^{-6}\,\mathrm{mol}\,\mathrm{l}^{-1}$) also had high inhibition effects on the collagenase activity. The bands of 72 kDa MMP2 and 92 kDa MMP9 were clearly reduced as compared with the control group.

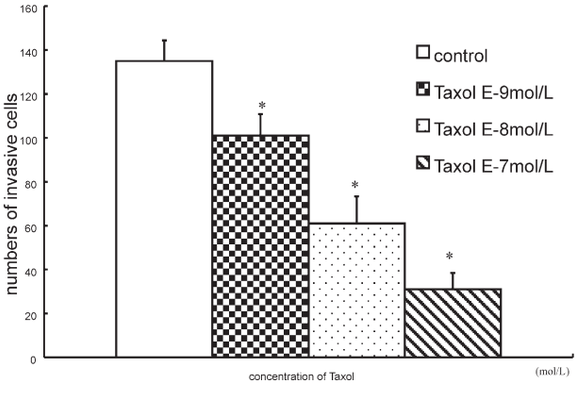

FIGURE 3 Anti-invasive effect of taxol on B16BL6. $^{*}P < 0.05$.

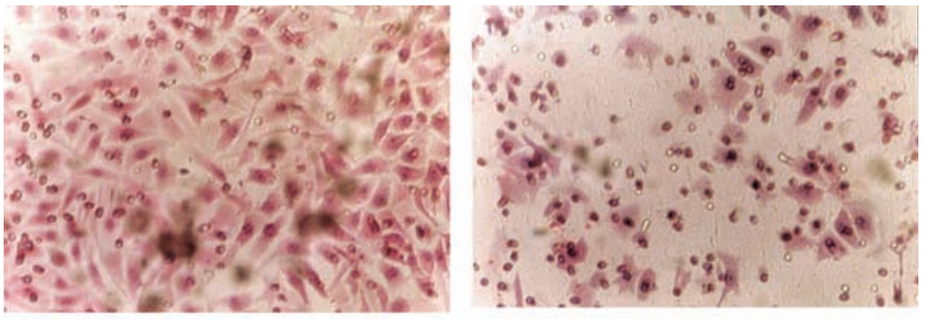

FIGURE 4 Anti-invasive effect of taxol on B16BL6. (A) Control, (B) taxol, 10^{-8} mol l^{-1}.

Inhibitory Effects on Migration

The migration potential of B16F10 cells exposed to taxol (10^{-8} mol l^{-1}) and CPT (10^{-8} mol l^{-1}) is given in Fig. 6. Taxol and CPT showed almost the same inhibition effect on the percentage of cell locomotion (22 ± 0.13%, 29 ± 0.16% vs 47 ± 0.17%) and the velocity of migrating cells (0.24 ± 0.02 μm h^{-1}, 0.25 ± 0.03 μm h^{-1} vs 0.29 ± 0.06 μm h^{-1}).

The Anti-angiogenesis Effect of Taxol and CPT in CAM Model

In the CAM model, taxol and CPT showed strong anti-angiogenesis effects (Fig. 7). The avascular area is larger than that of the control group.

DISCUSSION

Cancer metastasis is the greatest obstacle for successful tumor treatment. Although advances have been made in conventional tumor therapies, the majority of cancer deaths still result from metastatic disease. Melanoma B16BL6 and B16F10 are two highly invasive cell lines and they also have a high collagenase activity as compared with their parental cell line B16 [31]; therefore, they are widely used in metastasis studies *in vitro* and *in vivo* [32–35]. In this study, we used two highly metastatic cell lines, B16BL6 and B16F10, to investigate the anti-metastasis activity of taxol and CPT. It has been documented that the cellular features of these cell lines, such as adhesion to ECM, invasion, metalloproteinases and migration, are correlated with these cell lines forming secondary tumors *in vivo* [36].

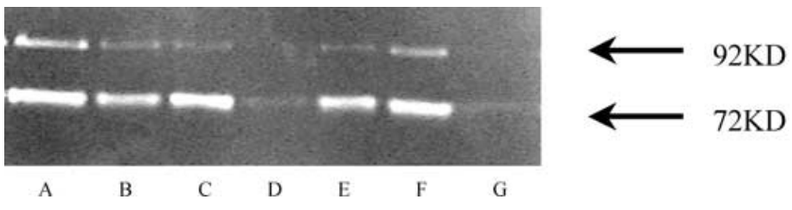

FIGURE 5 Secretion of type IV collagenases from HT1080 cells. (A) Normal control; (B, C, D) taxol, 10^{-10}, 10^{-8}, 10^{-6} mol L^{-1} respectively; (E, F, G) CPT, 10^{-10}, 10^{-8}, 10^{-6} mol l^{-1} respectively.

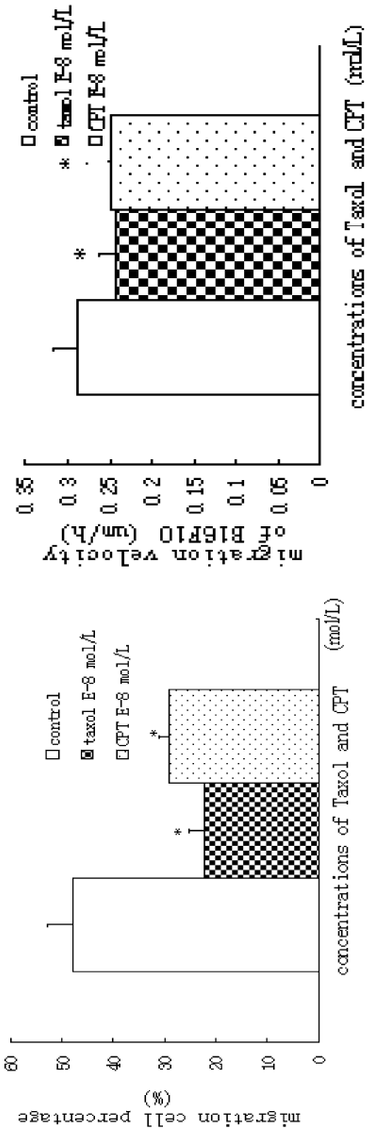

FIGURE 6 Anti-migration effect of taxol and CPT on B16F10.*$P < 0.05$.

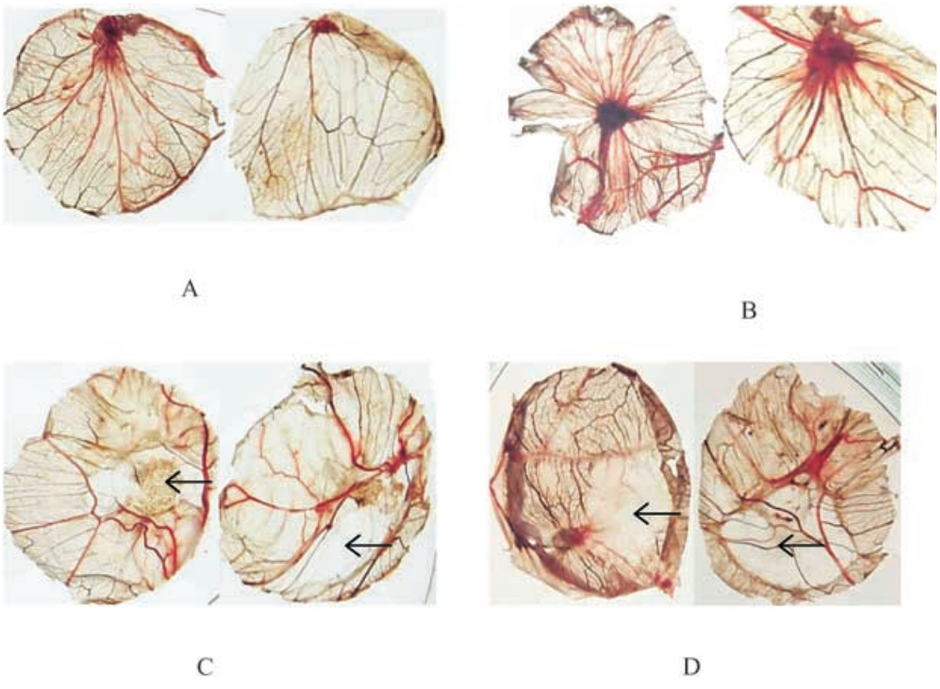

FIGURE 7 Anti-angiogenesis effect of taxol and CPT in the CAM model. (A) Normal; (B) bFGF, 200 ng; (C) taxol, 50 μg; (D) CPT, 50 μg. The arrow shows the avascular area caused by taxol and CPT respectively.

The adhesion of these melanoma cells to components of the ECM, such as fibronectin and laminin, facilitates metastasis to specific tissues. This study showed that taxol and CPT could inhibit the adhesive activity of B16F10 to fibronectin and laminin.

Metastatic tumor cells can show preferential attachment to the major collagen class, type IV collagen, of basement membranes. The degradation of the ECM and basement membrane components by tumor cells is an essential prerequisite for invasion and metastasis. Among the enzymes involved in this degradation, matrix metalloproteinases (MMPs) are a family of structurally related enzymes that are capable of degrading proteins of the extra-cellular matrix. The 72 kDa type IV collagenase is a neutral metalloproteinase capable of degrading type IV collagen which is a major component of basement membrane. The enzyme is mostly secreted into an extracellular milieu in a proenzymatic form. Many metastatic tumor cells, such as HT-1080 used in our study, can produce enzymes capable of cleaving the major proteoglycan of basement membranes. The production of type IV collagenases in metastatic tumor cells is well correlated with metastatic potential *in vitro* and *in vivo*. Taxol and CPT can reduce the ability of the collagenases secretion of HT1080 significantly in our study.

Tumor invasion and metastasis is a complex process. Most tumor cells possess the necessary cytoplasmic machinery for active locomotion. Inhibition of tumor cell motility may inhibit tumor cell invasion and metastasis. Taxol and CPT clearly can reduce the migration cell percentage and the velocity of B16F10 cells.

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is necessary for both growth and metastasis of solid tumors [37–39]. It is a complex process involving a series of sequential steps [40] including the degradation of the basement membrane of vessels, migration of endothelial cells towards angiogenic stimuli, proliferation of

endothelial cells, formation of capillary lumina and maturation of blood vessels. Angiosuppressions may be an inhibition factor of one or more of these steps and they are recognized as potential useful drugs in cancer metastasis. In this CAM model, bFGF was used as positive control stimulating angiogenesis. The results demonstrated that taxol and CPT exhibit a significant anti-angiogenesis effect *in vivo*.

In summary, our *in vitro* and *in vivo* results demonstrated that taxol and CPT could reduce the metalloproteinase secretion of HT1080 and exhibit an anti-angiogenesis effect in the CAM model. In a transwell chamber assay, taxol showed a strong anti-invasive effect on B16BL6 cells while CPT did not have this effect. This phenomenon is coincident with Liu's report and the difference in mechanisms deserves further studies. Taxol and CPT can inhibit the migration of B16F10 cells, and inhibit the adhesion of B16F10 to fibronectin and laminin. In Liu's report [24], CPT did not have the antimigration effect on B16BL6 cells; the difference may be owing to the different test method and cell lines used in our study. These effects possibly contribute to their anti-metastatic action. More *in vivo* and *in vitro* models should be explored to expose the further mechanisms of the two drugs in their clinical use.

EXPERIMENTAL

Cell Culture and Culture Conditions

Mouse melanoma cells (B16BL6, B16F10), and human fibrosarcoma HT1080 cell lines maintained at the Department of Pharmacology I, Institute of Materia Medica, CAMS & PUMC, were used in this study. Taxol and CPT were kindly provided by Professor Qi-Cheng Fang and Professor Xian-Dao Pan, respectively. Cells were cultured in a RPMI 1640 medium containing 10% bovine serum, streptomycin ($100\,\mu g\,ml^{-1}$), penicillin ($100\,U\,ml^{-1}$) at 37°C in a humidified atmosphere with 5% CO_2.

Adhesion Assay

Fibronectin or laminin (2.0 μg) was applied on 96-well plates and dried at room temperature; 20 μl RPMI 1640 containing 2% BSA was used to coat the plate at 37°C for 1 h, then it was rinsed with PBS (3 ×). Some 5×10^4 cells pre-treated with taxol (10, $0.1\,nmol\,l^{-1}$) and CPT (10 and $0.1\,nmol\,l^{-1}$) respectively for 24 h were added to each well and incubated for 1.5 h; PBS was then used to rinse the non-adhesive cells. 100 μl MTT ($0.4\,mg\,ml^{-1}$) was added to each well and incubated for 4 h. The absorbance value was then measured at 540 nm.

Invasion Assay

The invasion activity of tumor cells was determined by using a transwell chamber according to the methods described previously [41]. Briefly, poly(vinylpyrrolidone)-free polycarbonate filters with an 8.8 μm pore size were pre-coated with 5 μg of fibronectin in a volume of 10 μl on the outer side and dried at room temperature. Matrigel was diluted to $500\,\mu g\,ml^{-1}$ with cold phosphate-buffer saline (PBS), 10 μl were applied to the inner side of the filter and dried at room temperature. Tumor cells at log phase were harvested with $1\,mmol\,l^{-1}$ EDTA in PBS, washed twice and re-suspended to a final concentration of $2 \times 10^6\,ml^{-1}$ in bovine serum albumin (BSA). Cell suspensions (100 μl) were added to the upper compartment and taxol and CPT at different concentrations were added at the same time and the filter chamber was incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere. The filters were fixed with methanol for 1 min and then stained with hematoxylin for 3 min and eosin for 30 s. The cells

on the upper side of the filter were removed by wiping them with a cotton swab. The cells that had invaded through matrigel and reached to the reverse side were counted under a microscope in five fields at a magnification of $\times 400$. Each assay was preformed in triplicate.

Gelatin Zymography for the Determination of Collagenase Activity

HT1080 cells (2.5×10^5) were cultured overnight in 24-well plates. The cells were rinsed with PBS and incubated in serum-free RPMI 1640 medium. One hour later, the cells were rinsed twice again and incubated in serum-free RPMI1640 medium with taxol and CPT at different concentrations. After 24 h incubation, the supernatant was collected by centrifugation at 2000*g* for 10 min at 4°C. The cell-free medium was used to detect the gelatinase activity. Gelatin zymography was performed according to the method reported by Heussen and Dowdle [42] with some modifications. Briefly, samples were applied to SDS-polyacrylamide gels (10%, w/v) containing 0.1% (w/v) gelatin. After electrophoresis the gels were rinsed twice (30 min and 1 h each) in 2.5% Triton X-100 to remove SDS, and incubated for 16 h at 37°C in an incubation buffer (50 mmol l^{-1} Tris–HCl, pH 7.5, containing 10 mmol l^{-1} $CaCl_2$, 1 μmol l^{-1} $ZnCl_2$, 200 mmol l^{-1} NaCl). The gelatin gels were stained by 0.1% Coomassie brilliant blue R-250 solution containing 10% acetic acid and 10% isopropanol, bleaching in the solution containing 10% acetic acid and 10% isopropanol and then dried. Enzyme-digesting regions were identified as white bands against a blue background and determined by computerized scanning densitometry using an UVI system (UK).

3D Collagen Preparation and Tumor Cell Migration Assay

3D collagen matrices were prepared as described previously [43]. Briefly, 50,000 cells were mixed with a collagen solution consisting of 1.67 mg ml^{-1} type I bovine dermal collagen (Vitrogen 100) in Earle's modified Eagle's minimal essential medium (MEM) adjusted to pH 7.4; then a 100 μl cell suspension was poured into a small chamber and allowed to polymerize for 20–30 min (37°C, 5% CO_2).

Cell migration was documented as described in the literature [43]. Briefly, cells in the chamber were visualized on a conventional inverted microscope. A time-lapse video-microscope was used to record the movement. Then the paths of individual cells were reconstructed from the recorded films by computer-assisted cell tracking. The time-lapse video movie was displayed on a computer screen. From the first frame of the time-lapse sequence 30 cells were randomly selected. Subsequently, the movement of each cell was individually monitored by a trackball. Every 2 min (real time of the time-lapse film) the $x–y$ coordinates of the screen-pointer were registered by the computer. Plotting the $x–y$ data on the screen reconstructed the paths of individual migrating cells. The vector length between two $x–y$ coordinates was used as a direct measure of distance. Analyzing the corresponding vectors in each of the 30 paths simultaneously gave access to the percentage of migrating cells in a population.

Chicken Chorioallantoic Membrane (CAM) Model for Angiogenesis Study

The CAM assay was carried out according to the procedure described previously [44] with a minor modification; a 9-day embryo was used in this experiment. Briefly, a 0.5×1.5 cm pole right inferior the embryo head was chiseled carefully and the chorioallantoic membrane was exposed clearly. 50 μg of taxol and CPT was suspended respectively in 10 μl of methylcellulose which was then air-dried and a 3 mm diameter piece implanted on an

avascular zone of the CAM. After 3 days, the embryo was fixed by acetone: formaldehyde (3:1) and the CAM was clipped and examined.

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