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Anti-Angiogenic Effect of Silymarin on Colon Cancer LoVo Cell Line

Shung-Haur Yang, M.D.,* Jen-Kou Lin, M.D.,Ph.D.,† Wei-Shone Chen, M.D.,Ph.D.,† and Jen-Hwey Chiu, M.D.,Ph.D.‡,§,[1]

**Institute of Clinical, ‡Traditional Medicine, National Yang-Ming University; and †Division of Colon and Rectal Surgery, §Division of General Surgery, Department of Surgery, Taipei Veterans General Hospital, Taipei, Taiwan, Republic of China*

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Objective. **This study was designed to evaluate the anti-angiogenic effect of silymarin (SM) and its major pure component silibinin (SB), and also thalidomide (TH).**

Materials and methods. **A modified *in vitro* system using a coculture of endothelial (EA.hy 926) and colon cancer (LoVo) cell lines was adopted in this study.**

Results. **In cytotoxicity assay, SM/SB/TH concentrations causing 20% (IC_{20}) inhibition of cellular growth were 41.8 μg/ml/0.22 mM/0.088 mM for EA.hy 926 cells, and 16.1 μg/ml/0.12 mM/0.099 mM for LoVo cells, respectively. All 3 drugs showed concentration dependent inhibition of migration and differentiation assay. The IC_{50} inhibiting chemotaxis migration of EA.hy 926 towards LoVo by SM/SB/TH was 1.15 μg/ml/0.66 μM/1.98 μM, respectively. In differentiation assay, SM/SB/TH inhibited *in vitro* capillary tube formation by 50% at 1.25 μg/ml/2.6 μM/6.3 μM, respectively. In an analysis of vascular endothelial growth factor secreted by LoVo cells, SM/SB/TH decreased 50% secretion at 6.52 μg/ml/6.6 μM/131.7 μM, respectively.**

Conclusion. **SM/SB has a strong anti-angiogenesis effect on the colon cancer cell line, and this might provide an alternative treatment option for anti-cancer treatment.**

Key Words: **Silymarin; silibinin; thalidomide; colon cancer; angiogenesis; VEGF.**

INTRODUCTION

Previously, many studies have confirmed that solid tumors stimulate angiogenesis, and angiogenesis is obligatory for tumor growth [1]. Based on the studies on microvessel density, angiogenesis was thought not only as an early and critical step in adenoma-cancer tumorigenesis of colorectal cancer [2] but also an independent prognostic factor [3–5]. Of the various cytokines involved in angiogenesis, vascular endothelial growth factor (VEGF) is the key mediator and also the most extensively investigated one in tumor angiogenesis [6, 7]. VEGF signaling is mediated by 2 endothelial cell-selective receptors, called VEGF-R1 and VEGF-R2 (Flt1 and Flk1/KDR, respectively). VEGF level was reported as a clinically useful marker for colorectal cancer progression and metastasis, independent of other markers [8].

Anti-angiogenesis has become one of the major issues of tumor treatment. Many chemicals with cancer prevention effect have been of interest in recent years. Of them, naturally occurring polyphenols are receiving more attention [9]. Silymarin (SM), the active extract of milk thistle, is a polyphenolic flavonoid antioxidant and has been used as a liver detoxicant for many years [10]. SM is a mixture of the flavonolignans, silidianin, silichristine, and silibinin (SB) (also called silybin), with the latter being the most biologically active [11]. Studies in various animals using different modes of administration showed that SM is nontoxic in acute tests, even at large doses, and it is nontoxic in subchronic and chronic tests, and does not show any side effects [12]; there is no known LD50 for SM in laboratory animals [13]. Recently, the anticarcinogenic effects of SM have been reported in mouse skin tumorigenesis models [14, 15] and in studies using several cancer cell lines [16, 17]. The anti-angiogenesis effect against prostate and breast cancer cell lines via decreasing secretion of VEGF has also been reported [18]. Based on these understandings, we hypothesize that SM has a similar anti-angiogenesis effect against colon

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[1] To whom correspondence and reprint requests should be addressed at Institute of Traditional Medicine, School of Medicine, National Yang-Ming University, 155, Sec. 2, Li-Nong St., Peitou, Taipei, 112, Taiwan. E-mail: chiujh@mailsrv.ym.edu.tw.

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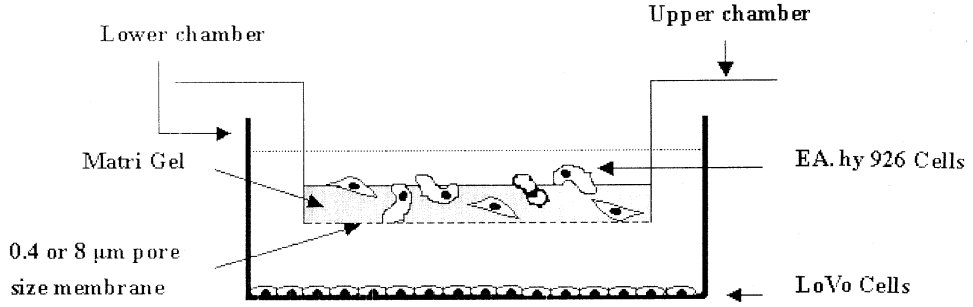

FIG. 1. Coculture system. Confluent LoVo cells are at lower chamber. EA.hy 926 cells and Matrigel are at upper chamber. Different pore size of upper chamber was used for migration assay (0.4 μm) or differentiation assay (8.0 μm), respectively.

cancer cells. Cytotoxic assay and modified *in vitro* systems using the coculture of endothelial and colon cancer cells are adopted in this study [19].

MATERIALS AND METHODS

Cell Lines and Cell Culture

The human endothelial cell line EA.hy 926 is a permanent human cell line that expresses highly differentiated functions characteristic of human vascular endothelium [20]. We used Dulbecco modified Eagle medium (DMEM) or serum-free OPTI (Life Technologies, Inc., Bethesda, MD). The full medium was DMEM with high glucose, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. LoVo colon cancer cells were obtained from American Type Culture Collection (Manassas, VA) and were cultured in the same medium as EA.hy 926.

Chemicals and Reagents

SM was tested simultaneously with SB to see if the effect is relevant with the crude extract form or its major pure compound. To be compared with SM, thalidomide (TH) was also tested because of its reported inhibitory effect on angiogenesis [21, 22]. SM and SB ($C_{25}H_{22}O_{10}$; M.W. 482.4) were purchased from Sigma Chemical Co. (St. Louis, MO). TH ($C_{13}H_{10}N_2O_4$; M.W. 258.2) was kindly provided by TTY Biopharm (Taiwan, ROC). All 3 drugs were dissolved with 2-hydroxypropyl-β-cyclodextrin (HBC) (RBI, Sigma, UK). The final concentration of HBC in mixture was 0.1%; controls were treated only with solvent. In preliminary experiments, the 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) test was used to calculate 20% inhibitory concentrations (IC_{20}) of all drugs against both cell lines. SM/SB/TH doses causing 20% (IC_{20}) inhibition of cellular growth were 41.8 μg/ml/0.22 mM/0.088 mM for EA.hy 926 and 16.1 μg/ml/0.12 mM/0.099 mM for LoVo cells, respectively. To reach minimal cytotoxic effect, 10 μg/ml for all 3 drugs were chosen as the maximal concentration limit for additional experiments. It equals 0.021 mM SB and 0.039 mM TH. Growth Factor Reduced Matrigel Matrix was purchased from Biocoat Inc. (Washington, PA). Transwell apparatuses (Costar Inc., Acton, MA) were used in coculture systems (Fig. 1). The IC_{50} of all drugs were calculated, as the results showed dose dependent curves in migration, differentiation, and inhibition on secreted VEGF assays.

Migration Assay

Transwell with the polycarbonate membrane (12 mm diameter, 8 μm pore size), which was soaked with 0.5% acetic acid overnight, was coated with 60 μl Matrigel (allowed to gel for 3 h as the upper chamber). After solidification, the wells were washed and then incubated for 30 min with DMEM. LoVo tumor cells were grown in the lower chambers, and at confluence, the medium was changed to serum-free OPTI medium for 24 h. Then, OPTI was replaced with DMEM with 0.5% FBS containing drugs in various concentrations. Separately, EA.hy 926 cells (5×10^4) suspended in DMEM with 0.5% FBS were plated on the upper chambers that were then mounted to the lower ones. Cell movement was evaluated towards the direction of LoVo cells. The cells were incubated at 37°C for 6 h, which was shown by preliminary experiments to be the best timing for harvest. After incubation, nonmigrated cells on the upper surface of the membrane were removed with cotton swab. The cells that had migrated through would be found attached on the undersurface of the membrane after fixation with 4% paraformaldehyde and stained with hematoxylin. They were counted with ImageAccess software (Imagic Co., Galttburgg, Switzerland) under a microscope.

Differentiation (*In vitro* Capillary Formation) Assay

Tube formation of EA.hy 926s in Matrigel was analyzed using the polycarbonate membrane (12 mm diameter, 0.4 μm pore size). The preparations, including LoVo cells in lower chambers, the membrane with resultant Matrigel, and EA.hy 926 cells in upper chambers were the same as the chemotaxis migration assay. After incubation for 48 h in the presence of cocultured LoVo cells, tube-like structures that formed in the gel were measured by the total length of capillary network with ImageAccess software. Values were expressed as the ratio over the control group.

Secreted VEGF in Culture Medium

For expression of angiogenic factors, VEGF was evaluated using the Enzyme-Linked Immunosorbent Assay (ELISA) kit per manufacturer instructions (human VEGF-A BioLISA, Bender MedSystems, San Brano, CA). LoVo cells (5×10^5) were cultured in DMEM with 2.0% FBS, with increasing concentrations of SM, SB, and TH. After 8-h incubation, the medium was collected for ELISA. The concentrations of VEGF in the medium were measured.

Statistics

The data in each experimental group were expressed as mean $\pm$ standard error of the mean (SEM). Significance was determined by using one-way analysis of variance followed by Bonferroni method or Wilcoxon signed rank test for between groups comparison.

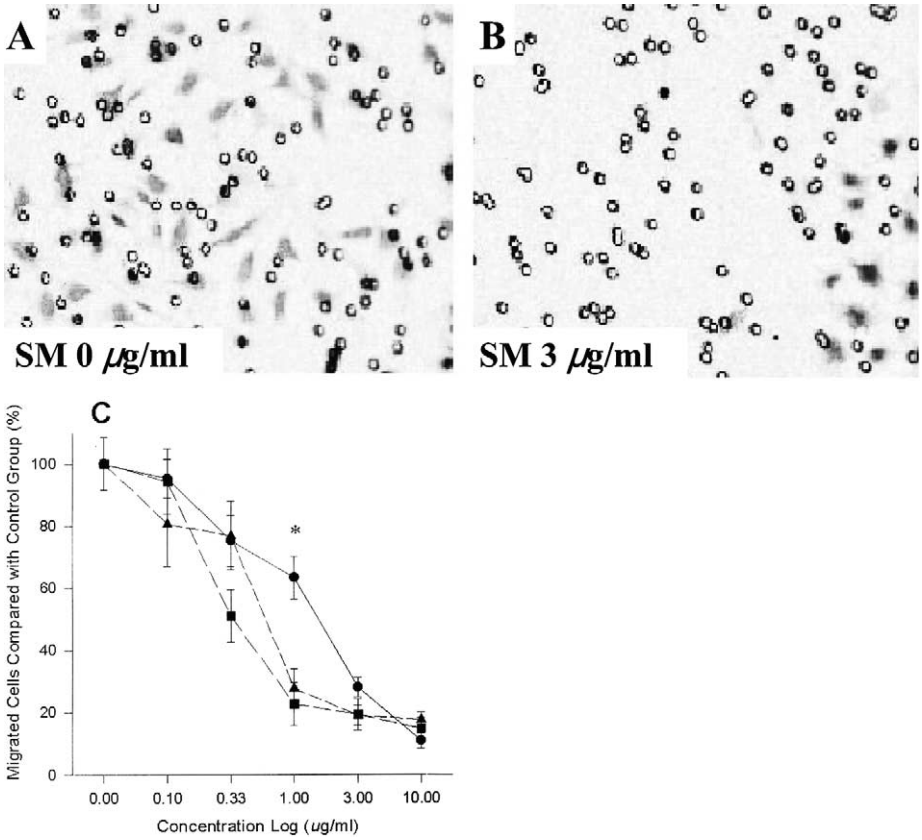

FIG. 2. Migration assay. There were significantly different amounts of migrated cells under the microscope at doses (A) 0 and (B) 3.0 μg/ml silymarin (SM). EA.hy 926 cells were distributed with 8 μm size pores (black dots) (original magnification $\times$40). (C) Effects of SM (●), silibinin (SB) (■), and thalidomide (TH) (▲) on chemotactic migration ($n = 6$). The curves of the 3 drugs showed dose-dependent inhibition on the amount of migrated cells. Values are reported as the percentage (mean + SEM) compared with the vehicle group (1086 $\pm$ 92.5 counted cells/membrane). The values of IC_{50} in the text were calculated according to the adjusted trend formula. Asterisks indicate $P < 0.05$.

RESULTS

Migration Assay

All 3 drugs have a suppressive effect on the chemotactic migration of endothelial cells toward the medium of LoVo (Fig. 2). Under the higher concentrations of drugs, the number of counted cells became fewer progressively. Interestingly, SB and SM had similar effects at lower and higher concentrations in this assay, but SB was more effective than SM at medium doses (1.0 μg/ml), with a significant difference. The curve trend of TH was the same with SM/SB, and it fluctuated between them. The IC_{50} of this migration assay were 1.15 μg/ml/0.66 μM/1.98 μM for SM, SB, and TH, respectively.

Differentiation (*In vitro* Capillary Formation) Assay

Under exposure to the 3 drugs, similar negative trends on the tubular forming ability of endothelial cells were noted (Fig. 3). The EA.hy 926 showed a dose-dependent response of inhibitory action of the capillary structure formation. The IC_{50} of this differentiation assay were 1.25 μg/ml/2.6 μM/6.3 μM for SM, SB, and TH, respectively. In this part, SM was almost as effective as SB. SB was more efficient than TH on the unit of molar concentration. All 3 drugs had a nearly 80% suppression effect at 10 μg/ml. Table 1 shows the calculated IC_{50} of drugs for migration and differentiation.

Secreted VEGF Content in Culture Medium

The detected VEGF corresponding to various concentrations of these agents is shown in Fig. 4. All 3 drugs showed a suppressive effect on the amount of secreted VEGF. At lower concentrations, TH had a similar effect as SM and SB. However, at higher concentrations, SM and SB curves became steeper downward, especially SB. The IC_{50} for secreted VEGF were 6.52 μg/ml/6.6 μM/131.7 μM for SM, SB, and TH, respectively.

DISCUSSION

In recent years, natural plant compounds had been receiving increasing attention about their anticarcino-

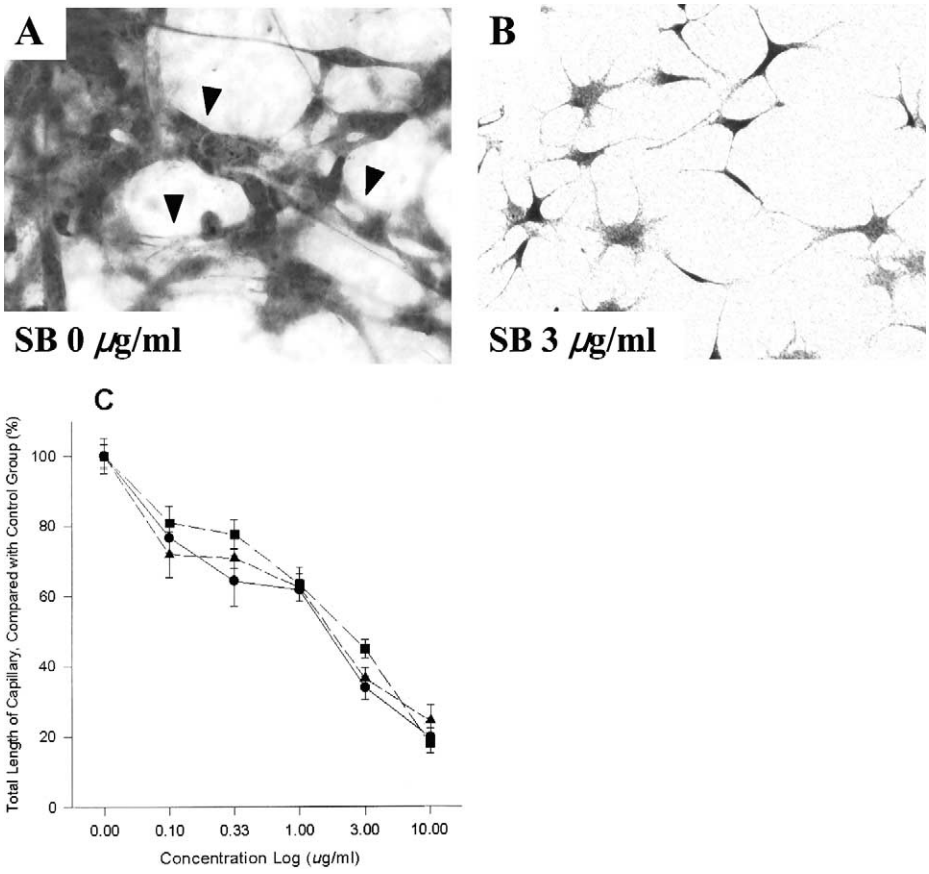

FIG. 3. Differentiation assay. There were significant differences in EA.hy 926 cell differentiation under the microscope. (A) At 0 μg/ml, silibinin (SB) cells are highly differentiated, elongated, and organized into vascular networks with hollow lumen (arrowheads). (B) While at 3 μg/ml, cells are distributed and alive but not organized into capillary (original magnification $\times$100.). (C) Effects of silymarin (SM) (●), SB (■), and thalidomide (TH) (▲) on capillary formation ($n = 6$). The curves of the 3 drugs show concentration dependent inhibition on the total length of capillary network. Values were expressed as the percentage (mean + SEM) compared with the vehicle group (89.6 $\pm$ 7.87 μm). The values of IC_{50} in the text were calculated according to the adjusted trend formula.

genesis ability. The phytochemical SM is one of the focuses because extensive investigation provided it the scientific basis of indications. It has been used with a high safety level for treating liver disease as a dietary supplement and treatment medicine for many years. As mentioned previously, Mukhtar and Agarwal had reported most of the works of anticancer and chemopreventive activity of SM [9]. Following their report of inhibition of VEGF and other angiogenic cytokines secreted by other cancer epithelial cells [18], this study reveals a similar phenomenon on colon cancer cells.

TABLE 1

Calculated IC_{50} of SM, SB & TH, on Inhibition of Endothelial Migration and Differentiation

Agents	Migration IC_{50}	Differentiation IC_{50}
SM	1.15 μg/ml	1.25 μg/ml
SB	0.66 μM	2.6 μM
TH	1.98 μM	6.3 μM

IC_{50}, Fifty percent inhibitory concentration; SM, silymarin; SB, silibinin; TH, thalidomide.

A cytotoxicity study shows significant dose-dependent survival inhibition of SM against both kinds of cells. The further selected dosage in subsequent angiogenesis studies were below the cytotoxicity IC_{20} of all drugs because we tried to avoid the direct cytotoxic effect by SM. The IC_{50} of SM for both phenotype assays was nearly the same (1.15 *versus* 1.25 μg/ml), and it means stable expression in the anti-angiogenic aspect. Inhibition of VEGF can explain their effects, but it is noted that IC_{50} for secreted VEGF was higher (6.52 μg/ml) than that of phenotype assays. This condition was also observed on SB and TH. It was speculated that SM might have multiple mechanisms to inhibit the angiogenesis. In the coculture model, drugs also contacted the EA.hy 926 in the upper chamber via the medium. Although the Matrigel coated membrane of the upper chamber may cause some gradient of drug concentration or secreted VEGF, between 2 chambers, there did exist the possibility of direct effects on these

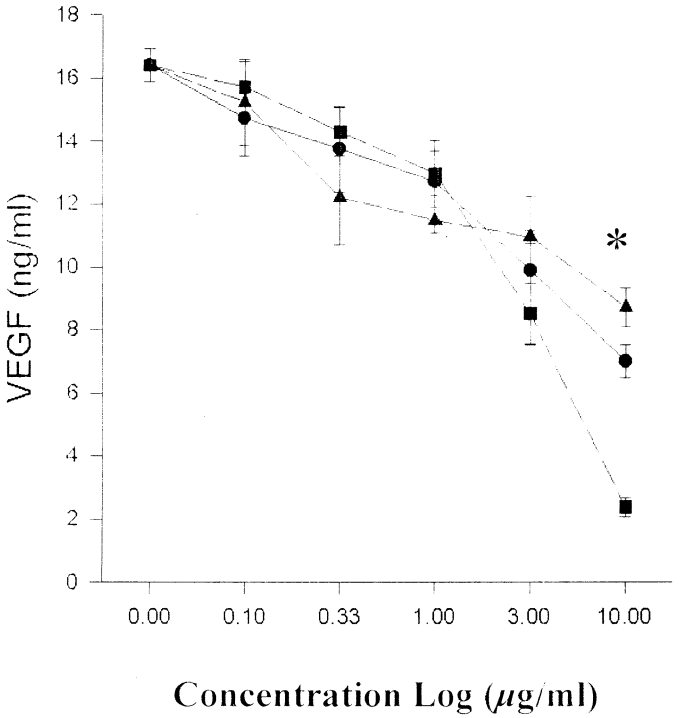

FIG. 4. Secreted vascular endothelial growth factor (VEGF) in medium. VEGF secretion was inhibited by silymarin (SM) (●), silibinin (SB) (■), and thalidomide (TH) (▲) in a dose-dependent manner. The values of IC_{50} in the text were calculated according to the adjusted trend formula. Asterisks indicate $P < 0.05$.

cells. Regarding this part, studies of direct effects on EA.hy 926 and LoVo cells are ongoing, both at ribonucleic acid and protein levels.

Angiogenesis is mainly influenced *in vivo* by paracrine angiogenic factors, and this coculture model mimics the microenvironment between cancer and endothelial cells. It has the advantage of real-time interaction and drug effects for both sides. The short half-life cytokine effects will not be lost in this model. Angiogenesis involves many complicated factors and procedures in sequence. Proliferation and migration of endothelial cells is mediated by VEGF-R2, while assembly into tubular structure is by VEGF-R1. Correlated with our design, the 2 phenotype assays represent the migration and differentiation into tubular structure, respectively. Based on current data, it is necessary to clarify the drug effect on EA.hy 926 cell expression of VEGF and its receptors in further studies.

Of the natural components of SM, SB is the most active, and it composes approximately 80% of SM. This pure compound component has the trend of better performance than SM in migration and secreted VEGF inhibition, and they are almost equal in that of differentiation. It can be assumed that most of the anti-angiogenesis effects of SM is from SB, which is consistent with the report of Bhatia *et al.* about its anticarcinogenic effects [23]. It will be one of the considerations if crude extract or pure compound will be used for further clinical application. The mechanism of SM anticarcinogenic effect has been related to inhibition of nuclear factor κ-B (NF-κB) [24], impairment of receptor tyrosine kinase [17], inhibition of cyclin-dependent kinase [16, 25], and as an antioxidant [15]. Interestingly, all these mechanisms also have been related to angiogenesis. The linking to inhibition of VEGF is a good beginning for study, but more laboratory work is needed to delineate the pathways of SM anti-angiogenic effect because the upstream of controlling VEGF production and secretion remains unknown.

Notorious for its teratogenic tragedy, TH regained its positive role in medicine in recent years. TH has been administered for certain autoimmune diseases, acquired immunodeficiency syndrome, leprosy, inflammatory bowel disease, and malignancies, especially myeloma [26]. Its effect is thought to be related to anti-angiogenesis, inhibition of VEGF [27], and inhibition of other cytokines, including tumor necrosis factor, and interleukins [28]. Some controversies existed about TH anti-angiogenesis effects because the results were speculated to be animal species related. Our coculture model using human cell lines could show its effects. For colon cancer, a pilot study of a combination therapy with TH and irinotecan had a remarkable absence of grade 3/4 gastrointestinal toxicities [29]. Calculated with the unit of molar concentration, TH is more toxic for endothelial cells than SB, equally toxic for tumor cells, but less effective for inhibiting angiogenesis. These results are compatible with the role of its immunomodulating and anti-inflammatory effect. Considering the cautious limit on women who will possibly give birth and the adverse effects, including constipation, sedation, skin rash, fatigue, and peripheral neuropathy [30], we think SM/SB has the prominent advantage for its safety. *In vivo* study is currently going to confirm the effects of these 3 drugs on real tissue.

CONCLUSION

SM/SB significantly inhibits the *in vitro* cell growth of colon cancer and endothelial cell lines. Under the influence of minimal toxicity (less than IC_{20}), SM/SB has a strong anti-angiogenesis effect on colon cancer cells, using *in vitro* coculture models (*i.e.,* induced endothelial cells migration and differentiation). VEGF secretion, the major angiogenic factor, could be decreased by SM/SB, and it should be one of the mechanisms explaining these observations but maybe not the only one. Based on the comparable results of SM and SB, we believe that most of the anti-angiogenesis effect of SM is from SB, and their effects are comparable, even better than TH.

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