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Inhibition of matrix metalloproteinase-2 secretion and invasion by human ovarian cancer cell line SK-OV-3 with lysine, proline, arginine, ascorbic acid and green tea extract

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Abstract

Aims: Based on the poor prognosis associated with ovarian cancer and reported anticancer properties of specific nutrients, we investigated the effect of a nutrient mixture (NM) containing lysine, proline, arginine, ascorbic acid and epigallocatechin gallate on human ovarian cancer cells SK-OV-3 by measuring: cell proliferation, modulation of matrix metalloproteinase (MMP)-2 and -9 expression, and cancer cell invasive potential.

Methods: Cell proliferation was evaluated by MTT assay, MMP activity by gelatinase zymography, and invasion through Matrigel.

Results: Human ovarian cancer cell growth was not significantly affected by the NM. Zymography demonstrated inhibition of MMP-2 secretion in a dose-dependent fashion with virtual total inhibition at 50 μg/mL NM concentration. Invasion of human ovarian cancer cells through Matrigel decreased in a dose-dependent fashion, with 90% inhibition at 500 μg/mL NM and 100% inhibition at 1000 μg/mL NM ($P < 0.0001$).

Conclusion: The combination of lysine, proline, arginine, ascorbic acid and green tea extract tested inhibited critical steps in cancer development and spread, such as MMP expression and invasion, indicating its potential as a treatment modality against ovarian cancer.

Key words: antitumor effect, matrix metalloproteinases, nutrient, ovarian cancer, SK-OV-3.

Introduction

Ovarian cancer is the seventh most common cancer among women and is attributed with the greatest number of female reproductive cancer-related deaths in the USA, with approximately 22 220 new cases and over 16 200 deaths estimated in 2005.[1]

Patient outcome is favorable in its early stages when the cancer has not spread outside of the primary organ; 5-year survival is 90–95%.[1] However, because there are no adequate means of early detection and ovarian cancer is asymptomatic in its early stages, in 71% of all diagnosed ovarian cancer cases metastasis has occurred and the cases are in the advanced stages.[2] Patient outcome greatly suffers once the cancer has metastasized to distant sites; 5-year survival is limited to 10%.[1]

The standards of treatment, including surgery, radiation therapy, chemotherapy, combination therapies and clinical trials have not addressed metastases, making these approaches ineffective at improving survival in the majority of diagnosed cases. Accordingly,

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mortality in ovarian cancer is approximately 65% of the incidence rate, indicating the need for new treatment approaches.[1]

Matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases that participate in normal tissue remodeling events, such as embryonic development, angiogenesis and wound healing, but are also associated with invasive and metastatic potential with aberrant increased expression. Degradation of the extracellular matrix (ECM) through the proteolytic cascade is key for tumor stromal invasion and subsequent metastasis. While a number of proteolytic enzymes are implicated in ECM degradation, MMPs, especially MMP-2 and MMP-9, have been identified as central to this process and to subsequent cancer progression.[3,4] The gelatinolytic activity of MMPs has also been found to correlate with the aggressiveness of tumor growth and invasiveness.[5]

Rath and Pauling[6] identified the destruction of the ECM as a precondition for cancer cell invasion, growth and metastasis and suggested interference through natural inhibitors of plasmin-induced proteolysis, such as lysine and its analogs. Previous studies have shown significant anticancer activity of the nutrient combination of lysine, proline, arginine, ascorbic acid and green tea extract against a number of cancer cell lines by blocking cancer growth, tissue invasion and MMP expression both *in vitro*[7–9] and *in vivo*.[10–12]

In the current study, we investigated the nutrient mixture (NM) of lysine, proline, arginine, ascorbic acid and green tea extract on human ovarian cancer cells *in vitro*, by measuring: cell proliferation, modulation of MMP-2 and MMP-9 and invasive potential.

Materials and Methods

Cell culture

Human ovarian cancer cells SK-OV-3 obtained from ATCC (American Type Culture Collection, Rockville, MD, USA) were grown in McCoy medium, supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL) in 24-well tissue culture plates (Costar, Cambridge, MA, USA). Cells were incubated with 1 mL of media at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO_2. At near confluence, the cells were treated with the NM, dissolved in media and tested at 0, 10, 50, 100, 500 and 1000 μg/mL in triplicate at each dose. Cells were also treated with phorbol 12-myristate 13-acetate (PMA) 200 ng/mL. The plates were then returned to the incubator. The cells were washed with phosphate buffered saline (PBS) and 500 μL of MTT (#M-2128, Sigma-Aldrich, St. Louis, MO, USA) 0.5 mg/mL in media was added to each well. Cell proliferation was evaluated 24 h following incubation with test reagents. Culture media components were purchased from Gibco (Grand Island, NY, USA).

MTT assay

Cell proliferation was evaluated by MTT assay. The MTT assay is a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrzolium salt [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and thus of cell viability. After the addition of MTT (0.5 mg/mL) the plates were covered and returned to the 37°C incubator for 2 h, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1 mL dimethylsulphoxide (DMSO), and absorbance was measured at 570 nm in a Bio Spec 1601, Shimadzu spectrometer (Schimadzu Scientific Instruments, Columbia, MD, USA). The OD_{570} of the DMSO solution in each well was considered to be proportional to the number of cells. The OD_{570} of the control (treatment without supplement) was considered to be 100%.

Gelatinase zymography

MMP activity in conditioned media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% Novex precast sodium dodecyl sulfate (SDS)-polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) in the presence of 0.1% gelatin under non-reduced conditions. Culture media (20 μL) mixed with sample buffer was loaded and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with Tris glycine SDS buffer, as described by the manufacturer (Novex). Samples were not boiled before electrophoresis. Following electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min at room temperature to remove SDS. The gels were then incubated at 37°C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM $CaCl_2$ at pH 8.0 and stained with 0.5% Coomassie blue R250 in 50% methanol and 10% glacial acetic acid for 30 min and destained. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobility of known proteins.

Matrigel invasion studies

Invasion studies were conducted using Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA) inserts in 24-well plates. Suspended in medium, human ovarian cancer SK-OV-3 cells were supplemented with nutrients, as specified in the design of the experiment and seeded on the insert in the well. Thus both the medium on the insert and in the well contained the same supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO_2 for 24 h. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with hematoxylin and eosin and visually counted under the microscope.

Morphology studies

Human ovarian cancer SK-OV-3 cells at various concentrations of NM were examined for changes in morphology using hematoxylin and eosin staining.

Composition of nutrient mixture

Stock solution of the NM prepared for testing was composed of the following: vitamin C (as ascorbic acid and as Mg, Ca and palmitoyl ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract 1000 mg (green tea extract derived from green tea leaves was obtained from US Pharma Laboratory (Somerset, NJ, USA). The certificate of analysis indicates the following characteristics: total polyphenol 80%, catechins 60%, epigallocatechin gallate (EGCG) 35% and caffeine 1.0%); selenium 30 mg; copper 2 mg; manganese 1 mg.

The composition of this NM was based on targeting different biological pathways involved in cancer progression and metastasis. For example, ECM integrity is dependent upon adequate collagen formation and its controlled enzymatic degradation. Therefore, the amino acids lysine and proline were included as the main components of collagen fibers and ascorbic acid, which are essential for their hydroxylation. Manganese and copper are essential cofactors in collagen and ECM formation. In addition, ascorbic acid has been shown to inhibit cell division and growth through production of hydrogen peroxide.[13] Green tea extract has been shown to be a promising agent in controlling angiogenesis, metastasis and other aspects of cancer progression.[14] N-acetyl cysteine has been observed to inhibit MMP-9 activity[15] and invasive activities of tumor cells.[16] Selenium has been shown to interfere with MMP expression and tumor invasion,[17] as well as migration of endothelial cells through ECM.[16] Because arginine is a precursor of nitric oxide (NO), any deficiency of arginine can limit the production of NO, which has been shown to predominantly act as an inducer of apoptosis, as seen in breast cancer cells.[18]

Based on the evidence available in the literature and our own research, we postulated that a combination of ascorbic acid, lysine, proline, green tea extract, arginine, N-acetyl cysteine, selenium, copper and manganese would work synergistically. For example, we found that a combination of ascorbic acid, lysine and proline used with EGCG enhanced the anti-invasive activity of 20 µg/mL EGCG to that of 50 µg/mL.[19] Thus, by including nutrients like N-acetyl cysteine, arginine, selenium, manganese and copper in addition to ascorbic acid, proline, lysine and EGCG, we could obtain significant reductions in cell invasion at a much lower concentration of individual components.

Statistical analysis

The results were expressed as the mean ± SD for the groups. Data was analyzed by the independent sample *t*-test.

Results

Cell proliferation study

NM with and without PMA 200 ng/mL showed a slight but insignificant effect on human ovarian cancer cell growth, as shown in Figure 1 (a,b).

Gelatinase zymography study

Zymography demonstrated only MMP-2 secretion. PMA (200 ng/mL) did not induce MMP-9 secretion. NM inhibited MMP-2 activity in a dose-dependent fashion with virtual total inhibition at 50 µg/mL concentration (Fig. 2a,b).

Invasion study

NM significantly reduced the invasion of human ovarian cancer cells SK-OV-3 through Matrigel in a dose-dependent fashion, with 90% inhibition at 500 µg/mL and 100% at 1000 µg/mL NM ($P < 0.0001$). See Figure 3.

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Morphology study

Hematoxylin and eosin staining showed no morphological changes, even at the highest concentration of NM (Fig. 4a–e).

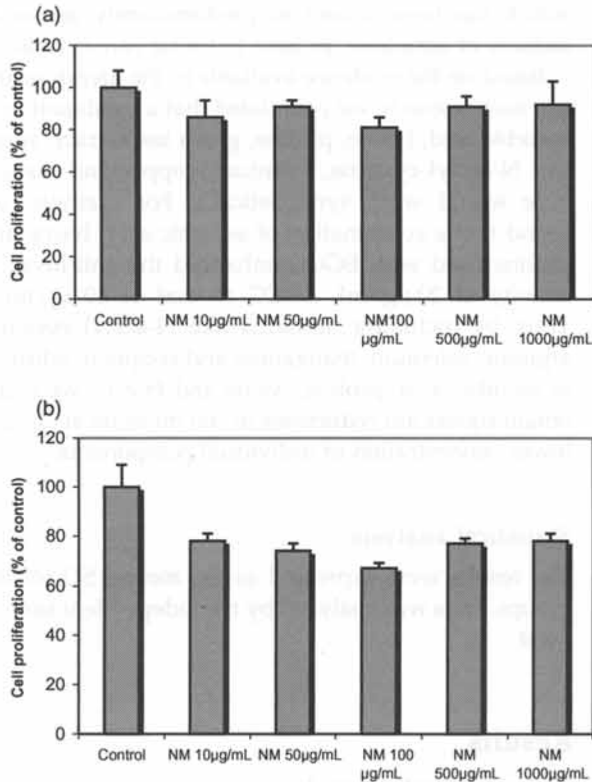

Figure 1 (a) Effect of nutrient mixture (NM) on cell proliferation of ovarian cancer SK-OV-3 cell line ($P = 0.366$). (b) Effect of NM on cell proliferation of phorbol 12-myristate 13-acetate (PMA; 200 ng/mL)-treated ovarian cancer SK-OV-3 cell line ($P = 0.02$).

Discussion

Metastasis to distant sites is dependent on the ability of the cells to penetrate the ECM and basement membrane and stimulate angiogenesis. Increasing the stability and strength of the connective tissue surrounding the cells can control matrix invasion. Optimization of synthesis and structure of collagen fibrils depends on hydroxylation of proline and lysine residues in collagen fibers. Ascorbic acid is essential for the hydroxylation of these amino acids.

Progression of malignant tumors can be impeded through the encapsulation of tumors by a decrease in matrix degradation accompanied by optimized ECM structure and its integrity. As a result, new treatment approaches are targeting universal pathomechanisms involved in cancer growth and invasion. Ascorbic acid, lysine, proline, manganese and copper support collagen formation. Rath and Pauling suggested targeting plasmin-mediated mechanisms with the use of nutritional components, such as lysine and lysine analogs.[6] Plasmin is formed upon cleavage of the zymogen plasminogen by plasminogen activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). Lysine prevents the activation of plasminogen into plasmin by tPA by binding to plasminogen active sites, and consequently prevents the plasmin-induced MMP activation cascade.[6] A recent study reported a seven-fold reduction in metastasis of transgenic mammary cancer in plasmin deficient mice.[20] *In vivo* and *in vitro* experimental models have highlighted the role of MMP-2 and MMP-9 in cellular invasion by disrupting ECM barriers and allowing tumors to spread locally and distantly.[21–23] In the current study, the dose-dependent inhibitory effect of the nutrient combination of lysine, proline, arginine, ascorbic acid and green tea extract on MMP-2 activity of

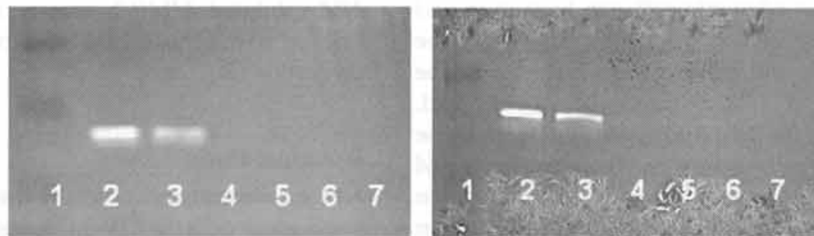

Figure 2 (a) Effect of nutrient mixture (NM) on ovarian cancer SK-OV-3 matrix metalloproteinase (MMP) expression. (b) Effect of NM on phorbol 12-myristate 13-acetate (PMA)-treated ovarian cancer SK-OV-3 MMP activity. The band relates to MMP-2 secretion. Lanes correspond as follows: 1, markers; 2, control; 3–7, NM 10, 50, 100, 500, 1000 µg/mL.

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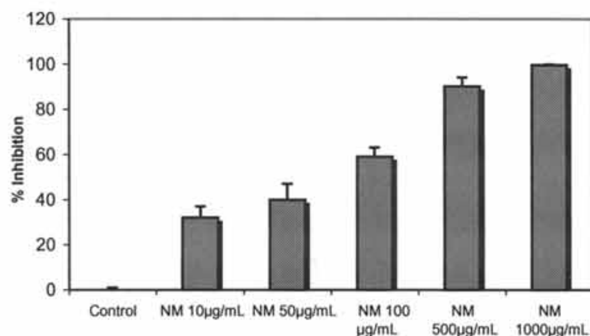

Figure 3 Dose-dependent inhibition of SK-OV-3 Matrigel invasion by NM with 90% inhibition at 500 μg/mL and total inhibition at 1000 μg/mL ($P < 0.0001$).

ovarian cancer cells SK-OV-3 was achieved at 50 μg/mL. However, with this cell line, a higher dose of the NM (1000 μg/mL) was required to completely block invasion of human ovarian cancer cells SK-OV-3 through Matrigel. Because inhibition of cancer cell invasion is dependent on blocking MMP secretion and enhancing ECM structure and integrity, as well as other targets, the higher dose required to block invasion is probably associated with ECM integrity.

The inhibitory effects of the individual nutrients tested have been reported in both clinical and experimental studies. Ascorbic acid has been reported to exert antimetastatic actions on malignant cell lines.[24–26] Moreover, depleted levels of ascorbic acid have been

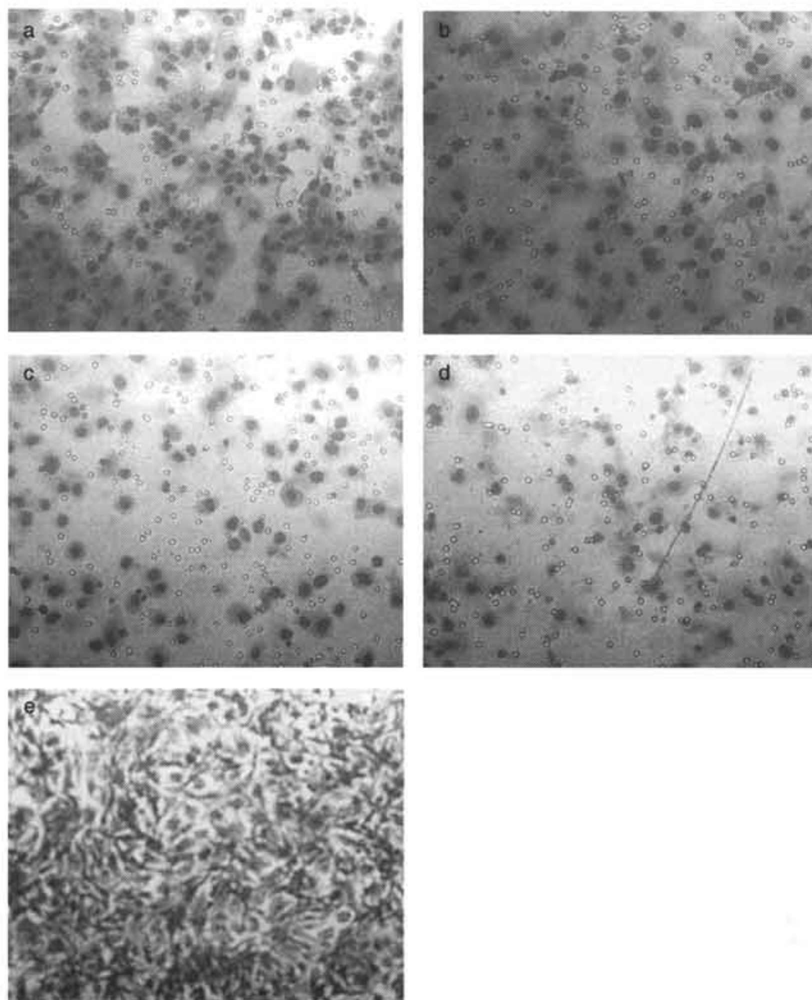

Figure 4 Hematoxylin and eosin staining showed no alterations in morphology after treatment of SK-OV-3 cells with nutrient mixture (NM) at various concentrations (10× magnification). (a) Control; (b) NM 50 μg/mL; (c) NM 100 μg/mL; (d) NM 500 μg/mL; (e) NM 1000 μg/mL).

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reported in cancer patients.[27–29] The protective effects of green tea extract have also been investigated; a recent cohort study conducted in China found that of 254 patients with histopathologically confirmed cases of epithelial ovarian cancer, 77.9% of those who consumed at least one cup of green tea per day reached 3-year survival *versus* 47.9% of non-green tea drinkers.[30] Another study found that (–) epigallocatechin –3-gallate (a constituent of green tea) inhibited ovarian cancer cell growth through the induction of apoptosis and cell cycle arrest.[31]

Our previous studies indicated that the synergistic anticancer effect of ascorbic acid, proline, lysine and green tea extract on several cancer cell lines in tissue culture studies was greater than that of the individual nutrients.[7] Additionally, in contrast to chemotherapy, morphological studies showed that the ovarian cancer cells were not affected even at the highest concentrations of NM, demonstrating that this formulation is non-toxic to cells. We found that the NM had no adverse effect on liver (albumin [A], globulin [G], A/G ratio, alkaline phosphatase and aspartate aminotransferase), heart (lactate dehydrogenase, creatine kinase and aspartate aminotransferase), kidney (creatinine [C], uric acid, blood urea nitrogen [BUN], BUN/C) functional serum enzymes, lipid profile (cholesterol and triglyceride levels), and histopathology (liver, kidney, heart, lung) when tested in adult female ODS rats gavaged with NM 30, 90 and 150 M daily for 7 days (unpublished data).

Through inhibition of MMP-2 secretion and invasion, our results suggest that the mixture of lysine, proline, ascorbic acid and green tea extract studied is an excellent candidate for therapeutic use in the treatment of ovarian cancer.

References

1. American Cancer Society. *Cancer Facts and Figures, 2005*. Atlanta, GA: American Cancer Society, 2005.
2. Auersperg N, Ota T, Mitchell GW. Early events in ovarian epithelial carcinogenesis: Progress and problems in experimental approaches. *Int J Gynecol Cancer* 2002; **12**: 691–703.
3. Michael M, Babic B, Khokha R *et al.* Expression and prognostic significance of metalloproteinases and their tissue inhibitors in patients with small-cell lung cancer. *J Clin Oncol* 1999; **17**: 1802–1808.
4. Naylor MS, Stamp GW, Davies BD, Balkwill FR. Expression and activity of MMPS and their regulators in ovarian cancer. *Int J Cancer* 1994; **58**: 50–56.
5. Kawamura K, Kamiya N, Suyama T *et al.* In situ gelatinolytic activity correlates with tumor progression and prognosis in patients with bladder cancer. *J Urol* 2004; **172**(4 Part 1): 1480–1484.
6. Rath M, Pauling L. Plasmin-induced proteolysis and the role of apoprotein (a), lysine and synthetic analogs. *Orthomol Med* 1992; **7**: 17–23.
7. Netke SP, Roomi MW, Ivanov V, Niedzwiecki A, Rath M. A specific combination of ascorbic acid, lysine, proline and epigallocatechin gallate inhibits proliferation and extracellular matrix invasion of various human cancer cell lines. *Res Comms Pharmacol Toxicol: Emerging Drugs* 2003; **2**: 37–50.
8. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. Antitumor effect of nutrient synergy on human osteosarcoma cells U-2OS, MNNG-HOS and Ewing's sarcoma SK-ES.1. *Oncol Rep* 2005; **13**: 253–257.
9. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. Synergistic effect of combination of lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate on colon cancer cell line HCT 116. *JANA* 2004; **7**: 40–43.
10. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. In vivo antitumor effect of ascorbic acid, lysine, proline and green tea extract on human colon cancer cell HCT 116 xenografts in nude mice: Evaluation of tumor growth and immunohistochemistry. *Oncol Rep* 2005; **13**: 421–425.
11. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. In vivo antitumor effect of ascorbic acid, lysine, proline and green tea extract on human prostate cancer PC-3 xenografts in nude mice: Evaluation of tumor growth and immunohistochemistry. *In Vivo* 2005; **19**: 179–183.
12. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. Modulation of N-methyl-N-nitrosourea induced mammary tumors in Sprague–Dawley rats by combination of lysine, proline, arginine, ascorbic acid and green tea extract. *Breast Cancer Res* 2005; **7**: R291–R295.
13. Kawakami S, Kageyama Y, Fujii Kihara K, Oshima H. Inhibitory effects of N-acetyl cysteine on invasion and MMP 9 production of T24 human bladder cancer cells. *Anticancer Res* 2001; **21**: 213–219.
14. Morini M, Cai T, Aluigi MG *et al.* The role of the thiol N-acetyl cysteine in the prevention of tumor invasion and angiogenesis. *Int J Biol Markers* 1999; **14**: 268–271.
15. Yoon SO, Kim MM, Chung AS. Inhibitory effects of selenite on invasion of HT 1080 tumor cells. *J Biol Chem* 2001; **276**: 20085–20092.
16. Maramag C, Menon M, Balaji KC, Reddy PG, Laxmanan S. Effect of vitamin C on prostate cancer cells in vitro: Effect on cell number, viability and DNA synthesis. *Prostate* 1997; **32**: 188–195.
17. Hare Y. *Green Tea: Health Benefits and Applications*. New York and Basel: Marcel Dekker, 2001.
18. Cooke JP, Dzau VJ. Nitric oxide synthase: Role in the genesis of vascular disease. *Annu Rev Med* 1997; **48**: 489–509.
19. Roomi MW, Ivanov V, Niedzwiecki A, Rath M. Synergistic antitumor effect of ascorbic acid, lysine, proline, and epigallocatechin gallate on human fibrosarcoma cells HT-1080. *Ann Cancer Res Ther* 2004; **12**: 148–157.
20. Almholt K, Lund LR, Rygaard J *et al.* Reduced metastasis of transgenic mammary cancer in urokinase-deficient mice. *Int J Cancer* 2005; **113**: 525–532.
21. Lopata A, Agresta F, Quinn MA, Smith C, Ostor AG, Salamonsen LA. Detection of endometrial cancer by determination

of matrix metalloproteinases in the uterine cavity. *Gynecol Oncol* 2003; **90**: 318–324.
22. Torng PL, Mao TL, Chan WY, Huang SC, Lin CT. Prognostic significance of stromal metalloproteinase-2 in ovarian adenocarcinoma and its relation to carcinoma progression. *Gynecol Oncol* 2004; **92**: 559–567.
23. Tamakoshi K, Kikkawa F, Nawa A *et al.* Characterization of extracellular matrix-degrading proteinase and its inhibitor in gynecologic cancer tissues with clinically different metastatic form. *Cancer* 1995; **76**: 2565–2571.
24. Naidu KA, Karl RC, Coppola D. Antiproliferative and proapoptotic effect of ascorbyl stearate in human pancreatic cancer cells: Association with decreased expression of insulin-like growth factor 1 receptor. *Dig Dis Sci* 2003; **48**: 230–237.
25. Anthony HM, Schorah CJ. Severe hypovitaminosis C in lung-cancer patients: The utilization of vitamin C in surgical repair and lymphocyte related host resistance. *Br J Cancer* 1982; **46**: 354–367.
26. Metz N, Lobstein A, Schneider Y *et al.* Suppression of azoxymethane-induced preneoplastic lesions and inhibition of cyclooxygenase-2 activity in the colonic mucosa of rats drinking a crude green tea extract. *Nutr Cancer* 2000; **38**: 60–64.
27. Koh WS, Lee SJ, Lee H *et al.* Differential effects and transport kinetics of ascorbate derivatives in leukemic cell lines. *Anticancer Res* 1998; **8**: 2487–2493.
28. Nunez C, Ortiz de Apodaca Y, Ruiz A. Ascorbic acid in the plasma and blood cells of women with breast cancer. The effect of consumption of food with an elevated content of this vitamin. *Nutr Hosp* 1995; **10**: 68–372.
29. Kurbacher CM, Wagner U, Kolster B *et al.* Ascorbic acid (vitamin C) improves the antineoplastic activity doxorubicin, cisplatin and paclitaxel in human breast carcinoma cells in vitro. *Cancer Lett* 1996; **103**: 183–189.
30. Zhang M, Lee AH, Binns CW, Xie X. Green tea consumption enhances survival of epithelial ovarian cancer. *Int J Cancer* 2004; **112**: 465–469.
31. Huh SW, Bae SM, Kim YW *et al.* Anticancer effects of (-)-epigallocatechin-3-gallate on ovarian carcinoma cell lines. *Gynecol Oncol* 2004; **94**: 760–768.

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