Inhibitory effect of vitamin K1 on growth and polyamine biosynthesis of human gastric and colon carcinoma cell lines

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Abstract. Gastric and colon cancers remain the leading cause of cancer mortality throughout the world. Since the gastrointestinal tract works in a constant link with the external environment, chemoprevention by dietary constituents could represent a possible approach to reduce cancer risk. Dietary vitamin K1 (VK1) has been shown to prevent the growth of many types of cancer cells. However, no data are available on possible different susceptibility to VK1 by gastric or colon neoplastic cell lines. Moreover, the exact mechanism of action of VK1 is still object of investigation, even if it has been reported that VK1 may induce cell cycle arrest and apoptosis. Therefore, molecules affecting cell growth such as the natural polyamines could be of interest in VK1 action. The aim of the present study was to investigate the effects of increasing concentrations of VK1 (from 10 to 200 μM) administered up to 72 h, on the cell proliferation and apoptosis of a gastric (HGC-27) and a colon (SW480) cancer cell line. Additionally, the polyamine biosynthesis and the MAPK pathway were also examined. VK1 treatments caused an inhibition of cell proliferation and an induction of apoptosis in both cell lines, with a concomitant significant decrease of the polyamine biosynthesis, increased phospho-ERK 1/2 expression was also observed. A different proliferative behavior and a different response to VK1 by gastric and colon cancer cells was evident, with colon cells showing a more pronounced susceptibility to VK1 action. VK1 is safe and without known toxicities in adult humans, consequently it could be effective in prevention and treatment of selected gastrointestinal neoplasms. Protocols based on the use of VK1, along with polyamine inhibitors and/or analogues, could represent a suitable alternative option for improving the efficacy of chemoprevention and treatment in future strategies for gastrointestinal cancer management.

Introduction

Gastric and colon cancers remain the leading cause of cancer mortality throughout the world. Overall, neoplasms of the gastrointestinal (GI) tract account for 25% of all neoplasms and for 9% of deaths from all causes in the world (1). Therefore, chemoprevention by dietary constituents is gaining interest as a possible approach to reduce cancer risk (2). The GI tract works in a constant link with the external environment and modifications in the diet may amplify or prevent exposure to carcinogenetic factors. In this context, identifying dietary components with antineoplastic activity and investigating their mechanisms of action are assuming importance in a new strategy of prevention for blocking or, at least, delaying GI cancer onset (2,3).

Among the substances naturally found in foods, evidence suggests that dietary vitamin K (VK) compounds are able to prevent the growth of many types of cancer cells, as demonstrated by studies performed in both *in vitro* and *in vivo* models (4-7).

VK is the family name for a series of fat-soluble compounds, classically associated to bone health and metabolism and in regulation of blood clotting (8). There are two structurally related natural forms of vitamin K: the vitamin K1 (VK1), phylloquinone and the vitamin K2 (VK2), menaquinone. The first is mainly found in green leafy vegetables, the latter is synthesized by the intestinal flora. Another compound, the vitamin K3 (VK3), menadione, is not considered a natural VK, but rather a synthetic analogue acting as a provitamin (8).

Most of data on the antineoplastic effects of VK family derives from studies performed on VK2 and VK3 and the treatment of hepatocellular carcinoma (9-11). As regards VK1, it has been proven to be effective in glioma and pancreatic cancer cell lines (12,13). However, in spite of its features as a nutritional factor, the potential of VK1 against neoplasms of the GI tract has received less or no attention so far (14). In addition, the exact mechanism of action of VK family in affecting the neoplastic growth is still object of investigation, even if it has

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been reported that VK compounds may alter the expression of some genes involved in cell cycle control and induce apoptosis in different cancer cell lines (15,16). A number of molecules, such as the natural polyamines: putrescine, spermidine and spermine, could be of interest in VK action. Polyamines are ubiquitous short-chain polycationic alkylamines actively involved in cell proliferation and differentiation. They interact with anionic macromolecules including DNA, RNA and protein, thereby modulating chromatin structure, gene transcription, translation and DNA stabilization (17). Moreover, it has been demonstrated that polyamines are also involved in signal transduction and in cell death pathway (18).

The rise in intracellular polyamine concentration, mostly occurring through an upregulation of the biosynthetic enzyme ornithine decarboxylase (ODC), correlates with increased cell proliferation and tumorigenesis (19). Thus, the mucosal polyamine levels can be considered markers for neoplastic proliferation (20). As a consequence, not only polyamine deprivation, but also impairment of polyamine uptake into neoplastic cells and modulation of the polyamine metabolic pathway, can represent a way of cancer chemoprevention and chemotherapy (21). Drugs that affect polyamine biosynthesis and catabolism have been extensively evaluated in both *in vitro* and *in vivo* studies as possible alternative tools for the management of cancer (22). Besides, some components of diet, particularly flavonoids, polyphenols and probiotics, have already been reported to reduce the hyperproliferative role of polyamines in GI neoplasms (23-25). On the contrary, the polyamine pathway as potential target for dietary VK has not been evaluated and no data are available on possible different susceptibility to VK1 by gastric or colon neoplastic cell lines.

In order to shed light on how VK1 could affect the growth of neoplasms originating from different GI tracts, the aim of the present study was to evaluate and compare the effects of increasing concentrations of VK1 on the cell proliferation and apoptosis of a gastric (HGC-27) and a colon (SW480) cancer cell line. Additionally, to investigate which mechanisms could be involved in affecting cell growth, the polyamine biosynthesis, as well as the phospho-ERK 1/2 (P-ERK 1/2) expression, were examined in the same cell lines. The latter signaling molecule has been linked to the regulation of cellular growth, apoptosis and chemoresistance (26).

Materials and methods

Cell culture conditions. HGC-27 cell line (neoplastic cells from undifferentiated carcinoma of the stomach) and human colon adenocarcinoma-derived SW480 cell line (low differentiated cells derived from colonic adenocarcinoma grade III-IV) were obtained from the Interlab Cell Line Collection (Genoa, Italy). As previously published (27), cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) and Leibovitz L-15 medium, respectively, supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin in a monolayer culture and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. At confluence, the cells were harvested by trypsinization and serially sub-cultured with a 1:4 split ratio. All cell culture components were purchased from Sigma-Aldrich (Milan, Italy).

Vitamin K1 treatment. HGC-27 and SW480 cells (25th-30th passage) were seeded at a density of $2x10^5$ cells/5 ml of supplemented Leibovitz L-15 and DMEM medium, respectively, in 60-mm tissue culture dishes (Corning Costar Co., Milan, Italy). After 24 h, to allow for attachment, the medium was removed and supplemented culture medium containing increasing concentrations of VK1 (10, 50, 100 and 200 μM) added to cells for 24, 48 and 72 h. Triplicate cultures were set up for each treatment and for the control, and each experiment was repeated 4 times. In the set of experiments investigating the role of ERK 1/2 on the VK1-induced apoptosis, the cells were treated with 20 μM MEK inhibitor (UO126) 2 h prior to 100- and 200-μM VK1 treatment.

Assessment of cell proliferation. After HGC-27 and SW480 cells had been cultured for 24, 48 and 72 h with increasing concentrations of VK1, the proliferative response was measured by colorimetric 3-(4,5 di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test.

To determine cell growth by colorimetric test, MTT stock solution (5 mg/ml in medium) was added to each dish at a volume of one tenth the original culture volume and incubated for 2 h at 37°C in humidified CO_2. At the end of the incubation period, the medium was removed, and the blue formazan crystals were solubilized with acidic isopropanol (0.1N HCl absolute isopropanol). MTT conversion to formazan by metabolically viable cells was monitored by spectrophotometer at an optical density of 570 nm (28).

Apoptosis. The apoptosis was measured by evaluation of Bax and Bcl-2 mRNA expression [using quantitative PCR (qPCR) method with SYBR1-Green dye] and protein expression of Bax, Bcl-2, caspase-3, caspase-9 and P-ERK 1/2 (using western blot analysis).

Cells were washed twice in phosphate-buffered saline (PBS) and then trypsinized and centrifuged at 280 x g. The cell pellets were re-suspended in 0.3 ml of pure distilled water and used for RNA extraction. Total cell RNA was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), following the manufacturer's instructions. Approximately 2 μg total cell RNA, extracted from both the control and treated cells, was used for cDNA synthesis. Reverse transcription (RT) was carried out in 20 μl of the final volume at 41°C for 60 min, using 30 pmol antisense primers for analyses of Bax, Bcl-2 and β-actin gene (28). The β-actin gene was utilized as an internal control and it was chosen as a reference housekeeping gene. Real-time PCRs were performed in 25 μl of final volume containing 2 μl of cDNA, Master Mix with SYBR-Green (iQ SYBR Green Supermix; Bio-Rad Laboratories, Milan, Italy) and sense and antisense primers for Bax, Bcl-2 and β-actin gene.

Real-time PCRs were carried out in a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories) using the following protocol: 45 cycles at 95°C for 3 min, 95°C for 10 sec, 55°C for 30 sec followed by a melting curve step at 65-95°C with a heating rate of 0.5°C per cycle for 80 cycles. The PCR products were quantified by external calibration curves, one for each tested gene. Curves were obtained with serial dilutions of known copy number of molecules (10^2-10^7 molecules). All expression data were normalized by dividing

the target amount by the amount of β-actin. The specificity of the PCR product was confirmed by gel electrophoresis.

For western blotting, HGC-27 and SW480 cells were collected and lysed on ice in RIPA buffer (Pierce Ripa buffer; Thermo Fisher Scientific, Rockford, IL, USA). After homogenization and centrifugation at 14,000 rpm for 15 min at 4°C, protein concentration was measured by a standard Bradford assay (Bio-Rad Laboratories). Aliquots of 50 µg of total proteins were separated in 4-12% pre-cast polyacrylamide gels (Invitrogen, Life Technologies, Carlsbad, CA, USA) and transferred onto a PVDF membrane (Bio-Rad Laboratories) with the Trans-Blot Turbo (Bio-Rad Laboratories). Bax, Bcl-2, caspase-3, caspase-9, P-ERK 1/2 and β-actin protein expressions were evaluated by 1:500 diluted Bax, Bcl-2 (D55G8), cleaved caspase-3 (Asp175), caspase-3 (3G2), cleaved caspase-9, caspase-9, phospho-p44/42 mitogen-activated protein kinase (MAPK) (ERK 1/2) (197G2), p44/42 MAPK (L34F12) and β-actin antibody, respectively (Cell Signaling Technology, Danvers, MA, USA). After overnight incubation, the membranes were further incubated with a horseradish peroxidase-conjugated goat secondary antibody (Bio-Rad Laboratories). The proteins were detected by chemiluminescence (ECL; Thermo Fisher Scientific). The densitometric analysis of each protein-related signal was obtained by using the Molecular Imager ChemiDoc™ (Bio-Rad Laboratories) and normalized against β-actin expression.

Polyamine biosynthesis. For the evaluation of polyamine levels after VK1 treatment, each cell culture pellet was homogenized in 700 µl of 0.9% sodium chloride mixed with 10 µl (200 nmol/ml) of the internal standard 1,10-diaminodecane (1,10-DAD). An aliquot of the homogenate was used to measure the total protein content. Then, to precipitate proteins, 50 µl of perchloride acid (PCA) 3M was added to the homogenate. After 30 min of incubation in ice, the homogenate was centrifuged for 15 min at 7,000 x g. The supernatant was filtered (Millex-HV13 pore size 0.45 µm; Millipore, Bedford, MA, USA) and lyophilized. The residue was dissolved in 300 µl of HCl (0.1 N). Dansylation and the extraction of dansyl-polyamine derivatives were performed as previously described (29). After extraction, aliquots of 200 µl were injected into a high-performance liquid chromatography system (UltiMate 3000; Dionex Corp., Sunnyvale, CA, USA) equipped with a reverse-phase column (Sunfire C18, 4.6x100 mm, 3.5 µm particle size; Waters Corp., Milford, MA, USA). Polyamines were eluted with a linear gradient ranging from acetonitrile-water (50:50, v:v) to acetonitrile (100%) for 30 min. The flow was 0.5-1.0 ml/min from 0 to 12 min and then set at a constant rate (1.0 ml/min) until the 30th min. The fluorescent intensity was monitored by a fluorescence detector (UltiMate 3000 RS; Dionex Corp.) with excitation at 320 nm and emission at 512 nm. Polyamine levels were expressed as concentration values in nmol/mg of protein.

ODC activity was measured with a radiometric technique that estimated the amount of $^{14}CO_2$ liberated from DL-[1-^{14}C]-ornithine (specific activity, 56.0 mCi/mmol; New England Nuclear, Boston, MA, USA) (30). The cell culture pellet (2-4x10^6 cells) was homogenized in 0.6 ml ice-cold Tris-HCl (15 mM, pH 7.5) containing 2.5 mM dithiothreitol, 40 µM pyridoxal-5-phosphate, and 100 µM ethylene diamine

tetra acetate and then centrifuged at 30,000 x g for 30 min at 4°C. An aliquot of supernatant (200 µl) was added to a glass test tube containing 0.05 µCi DL-[1-^{14}C]-ornithine and 39 nmol DL-ornithine. After incubation for 60 min at 37°C, the reaction was stopped by adding trichloroacetic acid (TCA) to a final concentration of 50%. $^{14}CO_2$ liberated from DL-[1-^{14}C]-ornithine was trapped on filter paper pre-treated with 40 µl NaOH (2N), which was suspended in a center well above the reaction mixture. Radioactivity on the filter papers was determined by a liquid scintillation counter (model 1219 Rackbeta; Pharmacia LKB Biotechnology AB, Uppsala, Sweden). ODC activity was expressed as pmolCO₂/h/mg of protein. Enzymatic activity was found to be linear within the range of 50-600 µg of protein (r2 = 0.99). The intra-assay and inter-assay variation coefficients (CV%) were 6 and 8%, respectively.

The effects of VK1 treatments on ODC mRNA levels were evaluated using the above described quantitative PCR method with SYBR1-Green dye and the appropriate primers.

Statistical analysis. Due to the non-normal distribution of the data, non-parametric tests were performed. Data were analyzed by Kruskal-Wallis analysis of variance and Dunn's multiple comparison test. All data are expressed as mean ± SEM. Differences were considered significant at P<0.05. The software package SigmaStat for Windows, version 3.00 (SPSS, Inc., San Jose, CA, USA) was used.

Results

Effects of VK1 on cell proliferation. Fig. 1 reports the effects of exposure of HGC-27 and SW480 cell lines to increasing concentrations of VK1 (namely 10, 50, 100 and 200 µM) for 24, 48 and 72 h. VK1 produced considerable antiproliferative effects in both cell lines.

In particular, VK1 administration caused a significant (P<0.05) reduction in the conversion of the MTT tetrazolium salt in HGC-27 cells compared to untreated control with concentrations starting from 100 µM after 48 and 72 h of treatment (-13 and -28%, respectively) (Fig. 1A).

In SW480 colon cancer cells this effect was more evident (-59%) and started earlier, after 24 h of 100 µM treatment (Fig. 1B). After 72 h, VK1 concentrations ≥50 µM were able to cause a significant and marked reduction (-62%) of cell proliferation (P<0.05).

Effects of VK1 on apoptosis. Fig. 2 shows the effects of increasing VK1 concentrations on apoptosis of the HGC-27 and SW480 cell lines, evaluated by Bax and Bcl-2 mRNA levels and expressed as Bax/Bcl-2 ratio.

HGC-27 cell line exhibited a significant (P<0.05) and dramatic increase in the Bax/Bcl-2 ratio at 48 h and with the highest concentrations of VK1 (100 and 200 µM) compared to control cells (+343 and +522%, respectively) (Fig. 2A). Similarly, in SW480 cells a significant (P<0.05) and even more dramatic increase in the Bax/Bcl-2 ratio was evident at 48 h and with the highest concentrations of VK1 (100 and 200 µM) compared to control cells (+750 and +500%, respectively) (Fig. 2B). The induction of apoptosis continued to be significant after 72 h of treatment with the concentration of 100 µM
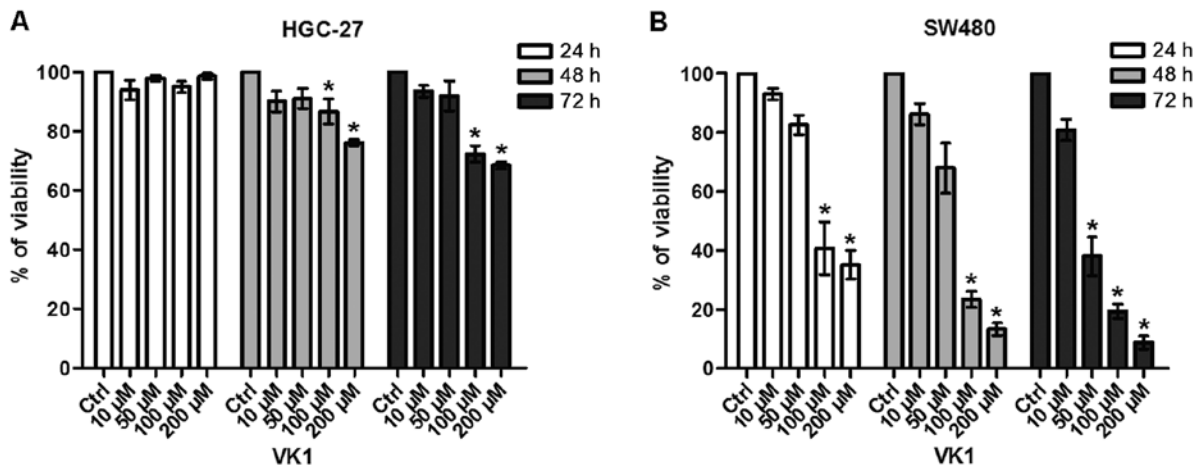
Figure 1. Proliferative response of HGC-27 and SW480 cell lines to vitamin K1 (VK1) treatment. Effects of increasing concentrations of VK1 (10, 50, 100 and 200 μM) on the conversion of MTT tetrazolium salt in HGC-27 (A) and SW480 (B) cell lines after 24, 48 and 72 h of treatment. All data represent the result of four different experiments (mean ± SEM). For each time of treatment, data were analyzed by Kruskal-Wallis analysis of variance and Dunn's multiple comparison test. *P<0.05 compared to control cells.
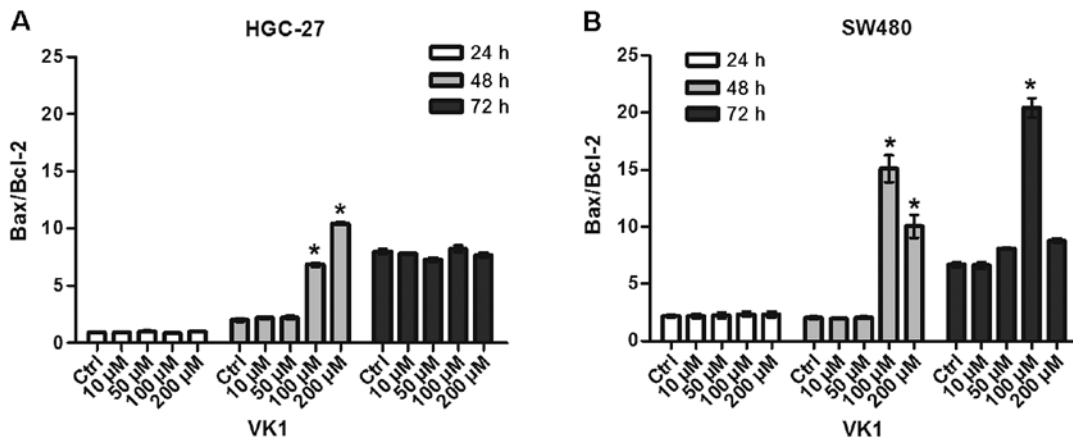

Figure 2. Apoptotic response of HGC-27 and SW480 cell lines to vitamin K1 (VK1) treatment. Effects of increasing concentrations of VK1 (10, 50, 100 and 200 μM) on the Bax/Bcl-2 mRNA level in HGC-27 (A) and SW480 (B) cell lines after 24, 48 and 72 h of treatment. All data represent the result of four different experiments (mean ± SEM). For each time of treatment, data were analyzed by Kruskal-Wallis analysis of variance and Dunn's multiple comparison test. *P<0.05 compared to control cells.

(+300%), while the Bax/Bcl-2 ratio dramatically decreased by 42% with the highest 200 μM concentration, probably as a consequence of VK1 toxic effect.

The protein levels of Bax, Bcl-2, caspase-3 and caspase-9 were evaluated by western blot analysis. In HGC-27 cells, no significant changes in Bax and Bcl-2 proteins or in Bax/Bcl-2 ratio occurred at all the times and concentrations used (data not shown). On the contrary, an increase in apoptotic proteins was observed only after 48 h of treatment in SW480 cells (Fig. 3A). More specifically, Bax protein significantly (P<0.05) increased by ~200% in cells treated with VK1 concentrations ≥100 μM compared to untreated cells (Fig. 3B). Bcl-2 significantly (P<0.05) decreased by 50% with VK1 concentrations ≥10 μM compared to control cells (Fig. 3C). A significant (P<0.05) 200% increase in the Bax/Bcl-2 ratio was observed with VK1 concentrations equal to 50 and 100 μM compared to untreated cells (Fig. 3D). With the highest 200 μM concentration, Bax/Bcl-2 ratio dramatically increased by 400% compared to control cells. Of note, the addition of UO126 blocked the

VK1 mediated induction of apoptosis in SW480 colon cancer cells.

As for the involvement of caspases in VK1-induced apoptosis, no significant cleavage of caspase-3, an executioner caspase, or caspase-9, an initiator caspase, was observed in the two cell lines. This evidence suggests that VK1 may induce apoptosis in these cells without activation of caspases (data not shown).

P-ERK 1/2 phosphorylation was evaluated by treating cells with increasing VK1 concentrations (from 10 to 200 μM) for 24, 48 and 72 h. No variation, at all the tested times was observed in P-ERK 1/2 in HGC-27 cells (data not shown). On the contrary, a dose-dependent significant (P<0.05) increase in P-ERK 1/2 was observed only after 48 h in SW480 cells with VK1 concentrations starting from 10 μM (+100%) (Fig. 4). When cells were pretreated for 2 h with 20 μM of the MEK inhibitor UO126 followed by VK1 treatment (100 and 200 μM) for 48 h, no induction of P-ERK 1/2 was observed. Therefore, as for Bax and Bcl-2 proteins, the addition of UO126 blocked
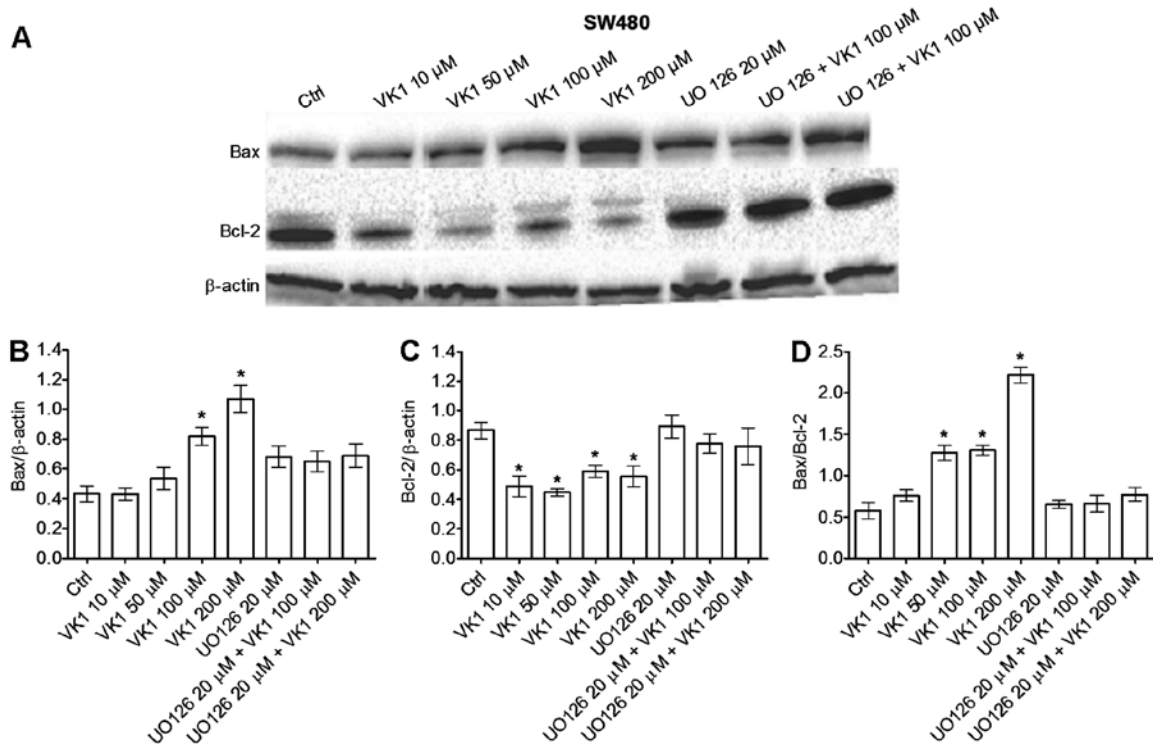
Figure 3. Western blot analysis of Bax and Bcl-2 in SW480 cell line after 48 h of vitamin K1 (VK1) treatment. The cells were exposed to increasing concentrations of VK1 (10, 50, 100 and 200 μM), 20 μM UO126 alone or in combination with 100 and 200 μM VK1. Immunoreactive bands were quantified using Quantity One Programme. The diagrams (B-D) show quantification of the intensity of bands, calibrated to the intensity of β-actin bands. All data represent the result of four different experiments (mean ± SEM). Data were analyzed by Kruskal-Wallis analysis of variance and Dunn's multiple comparison test. *P<0.05 compared to control cells.
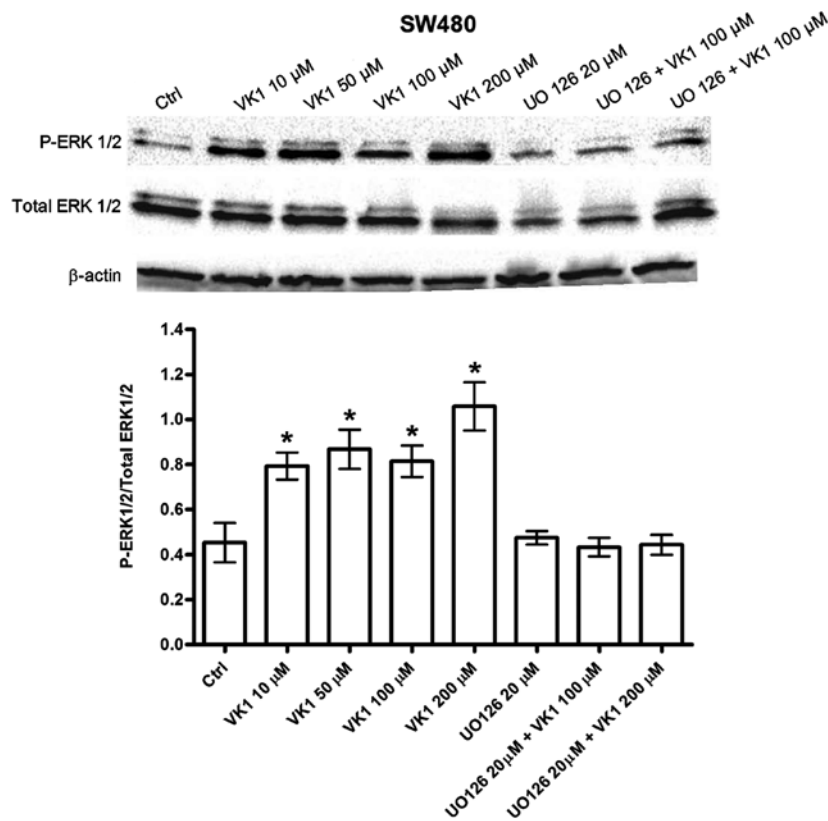

Figure 4. Western blot analysis of P-ERK1/2 and Total ERK1/2 in SW480 cell line after 48 h of vitamin K1 (VK1) treatment. The cells were exposed to increasing concentrations of VK1 (10, 50, 100 and 200 μM), 20 μM UO126 alone or in combination with 100 and 200 μM VK1. Immunoreactive bands were quantified using Quantity One Programme. The diagram shows quantification of the intensity of bands, calibrated to the intensity of β-actin bands. All data represent the result of four different experiments (mean ± SEM). Data were analyzed by Kruskal-Wallis analysis of variance and Dunn's multiple comparison test. *P<0.05 compared to control cells.

Table I. The polyamine profile.

HGC-27		Control	K1 10 μM	K1 50 μM	K1 100 μM	K1 200 μM
24 h	Put	0.17±0.02	0.14±0.02	0.14±0.03	0.11±0.02	0.14±0.02
	Spd	10.06±0.17	9.46±0.20	9.6±0.20	9.53±0.09	9.60±0.23
	Spm	17.26±0.93	17.81±0.54	17.03±1.0	17.31±0.68	17.56±0.51
	Total	27.50±0.79	27.42±0.76	26.77±1.7	26.94±0.79	27.39±0.73
48 h	Put	0.15±0.02	0.16±0.02	0.21±0.02	0.22±0.04	0.17±0.02
	Spd	9.92±0.29	8.73±0.39	7.6±0.40	5.81±0.22[a]	5.83±0.44[a]
	Spm	17.77±0.27	17.40±0.37	17.87±0.14	17.90±0.37	16.21±0.23
	Total	27.85±0.52	26.29±0.45	25.65±0.72	23.93±0.50[a]	22.21±0.54[a]
72 h	Put	0.18±0.01	0.11±0.02	0.16±0.03	0.07±0.01	0.04±0.01
	Spd	8.94±0.23	7.04±0.31	6.70±0.20	4.93±0.34[a]	4.25±0.22[a]
	Spm	19.01±0.28	16.77±0.83	16.49±0.81	15.17±0.31	14.90±0.37
	Total	28.13±0.55	23.93±0.46	23.35±0.64	20.17±0.44[a]	19.19±0.47[a]
SW480						
24 h	Put	2.10±0.19	1.33±0.09	0.98±0.12	0.60±0.10[a]	0.67±0.14[a]
	Spd	12.77±0.30	11.24±0.12	9.22±0.30	8.01±0.40[a]	7.72±0.31[a]
	Spm	12.45±0.30	11.62±0.19	10.30±0.30	8.83±0.16[a]	8.75±0.20[a]
	Total	27.32±0.77	24.80±0.45	20.02±0.78	17.44±0.61[a]	17.70±0.50[a]
48 h	Put	1.50±0.15	0.98±0.20	0.67±0.10	0.26±0.05[a]	0.25±0.09[a]
	Spd	9.40±0.28	8.20±0.37	7.10±0.39	5.95±0.35[a]	5.20±0.50[a]
	Spm	12.61±0.42	12.5±0.60	11.70±0.20	11.00±0.31[a]	11.30±0.23[a]
	Total	23.51±0.50	21.62±0.45	19.47±0.69	17.50±0.50[a]	16.75±0.53[a]
72 h	Put	1.68±0.11	1.45±0.12	1.53±0.10	0.80±0.12[a]	0.81±0.14[a]
	Spd	6.80±0.20	6.00±0.24	5.82±0.20	4.40±0.27[a]	4.27±0.20[a]
	Spm	12.10±0.23	11.82±0.19	11.90±0.36	11.1±0.15[a]	10.97±0.20[a]
	Total	20.58±0.50	19.27±0.48	19.25±0.63	16.30±0.44[a]	16.02±0.50[a]

Put, putrescine; Spd, spermidine; Spm, spermine; Total, total polyamine in HGC-27 and SW480 cell lines following administration of vitamin K1 (VK1) increasing concentrations (from 10 to 200 μM) after 24, 48 and 72 h. All data represent the results of four different experiments (mean ± SEM). For each time of treatment data were analyzed by Kruskal-Wallis analysis of variance and Dunn's multiple comparison test, [*]P<0.05 vs. control. Polyamines are in nmol/mg protein.

SW480 cells from VK1 mediated induction of apoptosis, thus suggesting a contribution of the MAPK pathway in this process.

Effects of VK1 on polyamine biosynthesis. Table I shows the polyamine profile in HGC-27 and SW480 cell lines following administration of increasing VK1 concentrations up to 72 h. VK1 treatment led to a decrease in the single and total polyamine contents in both cell lines. In detail, a significant (P<0.05) reduction in the spermidine and total polyamine content was observed in HGC-27 cells after 48 h (-41 and -14%, respectively) and 72 h (-45 and -28%, respectively) of exposure to VK1 concentrations ≥100 μM compared to control cells. In SW480 cells, the effect started after 24 h with VK1 concentrations ≥100 μM that induced a significant (P<0.05) decrease of all the single and total polyamine levels compared to control cells. Of note, the effect persisted after 48 and 72 h of treatment.

The effects of VK1 administration on ODC activity and ODC expression in HGC-27 and SW480 cell lines were studied at increasing concentrations (from 10 to 200 μM) up to 72 h. As shown in Fig. 5A and B, after 72-h administration, VK1 concentrations ≥100 μM significantly reduced (P<0.05) both ODC activity (-74%) and ODC expression mRNA (-50%) in HGC-27 cells compared to the control ones. Overall, the effect of reduction by VK1 in SW480 cells was more rapid, since ODC activity was significantly reduced (P<0.05) by 36% starting from 24 h of treatment with VK1 concentrations ≥50 μM compared to the untreated cells (Fig. 5C). Regarding ODC expression, 24-h administration of VK1 concentrations ≥100 μM caused a significant reduction (P<0.05) by 62.5% of ODC mRNA level compared to untreated cells (Fig. 5D). Notably, the significant effect persisted up to 72 h.

Discussion

Along with the proven effects on bone metabolism and blood clotting, compounds belonging to VK family have also been proposed as antineoplastic factors. However, the majority of
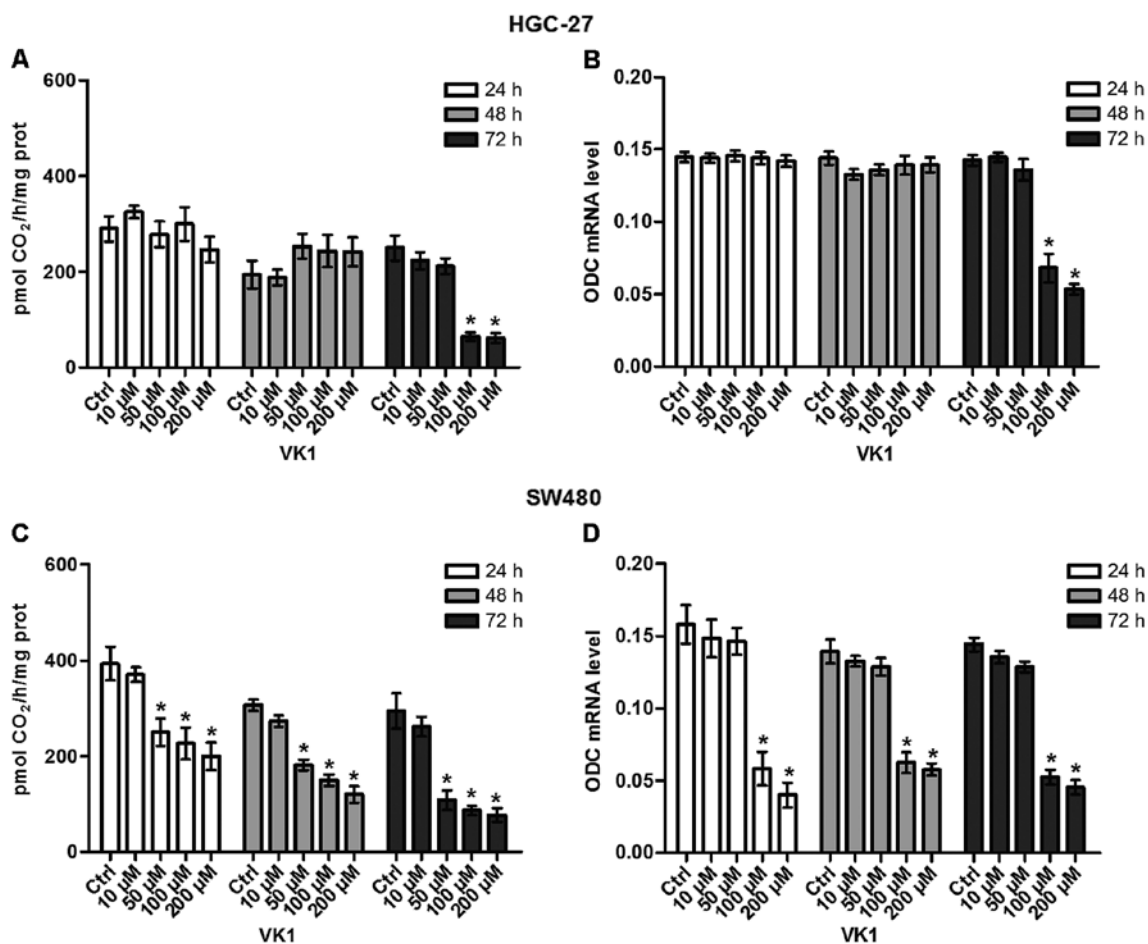
Figure 5. Ornithine decarboxylase (ODC) activity and mRNA level of HGC-27 and SW480 cell lines in response to vitamin K1 (VK1) treatment. Effects of increasing concentrations of VK1 (10, 50, 100 and 200 μM) on the ODC activity and mRNA level in HGC-27 (A and B) and SW480 (C and D) cell lines after 24, 48 and 72 h of treatment. All data represent the result of four different experiments (mean ± SEM). For each time of treatment, data were analyzed by Kruskal-Wallis analysis of variance and Dunn's multiple comparison test. *P<0.05 compared to control cells.

data on this issue derives from studies performed on VK2 and VK3 and the exact mechanisms of action are still not completely elucidated (6,31,32).

It has been reported that VK1 can inhibit survival and induce apoptosis in pancreatic cancer cell lines (33); it is also able to enhance sorafenib-induced growth inhibition in human hepatocellular carcinoma and malignant glioma (12,34), but scarce data are available on its potential against GI neoplasms (14). Hence, one of the aims of the present research was to compare the effects of VK1 administration on growth of two different human GI cancer cell lines, one originating from undifferentiated carcinoma of the stomach (HGC-27), the other from colon adenocarcinoma (SW480) paying attention to the involvement of polyamine biosynthesis.

The main finding of the present study is that VK1 administration not only reduced cell proliferation and induced apoptosis in both HGC-27 and SW480 cells, but also caused a significant decrease in the polyamine biosynthesis. Even if the response to increasing concentrations of VK1 shown by the two GI cancer cell lines was very similar, some peculiar differences must be underlined.

Firstly, as regards cell proliferation, VK1 administration to HGC-27 cells with concentrations ≥100 μM significantly reduced the MTT conversion after 48 h. In SW480 cells,

the inhibitory effect started rapidly after 24 h with the same concentration and, thus, demonstrating a more pronounced susceptibility of colonic cells to VK1 action.

Secondly, in accordance with MTT data, also the single and total polyamine levels decreased significantly after VK1 treatment in the same time-dependent manner. However, after 48 h of VK1 administration to HGC-27 cells, a significant reduction occurred only in the spermidine as well as total polyamine contents with the highest concentrations (100 and 200 μM). In SW480 cells, all the single and total polyamines significantly decreased already after 24 h of treatment with concentrations ≥100 μM.

Polyamines can be considered reliable markers of neoplastic proliferation, since abnormal hyperproliferative cells, such as those in neoplastic and pre-neoplastic tissue, exhibit very high requirements for these substances to sustain cell growth through elevated DNA, RNA and protein synthesis (18). Furthermore, it is known that polyamines influence the expression of various genes involved in cell proliferation, tumor invasion and metastatis (35). There is a vast body of literature describing the relationship between increased levels of polyamines and cancer, most of which suggests an association of induced polyamine biosynthesis with neoplasms (36). An important role is also played by ODC, a key-regulator enzyme

in the polyamine biosynthesis, that it is now considered as a true oncogene (37).

VK1 administration induced a significant decrease in ODC activity and expression in both the gastric and colon cancer cells. In HGC-27 cell line the decrease was significant at a late stage (after 72 h) with the highest concentrations (100 and 200 μM). On the contrary, in SW480 cells, a significant decrease in ODC activity occurred earlier with 50 μM of VK1 after 24 h of treatment and this effect persisted up to 72 h. A concomitant and significant inhibition of ODC gene expression was observed starting from 100 μM of VK1.

Data from the present study are in agreement with published studies performed using other nutritional components such as flavonoids, polyphenols and probiotics that have demonstrated how these factors are able to reduce the polyamine biosynthesis in different cancer cells, including the GI ones (38).

Functionally, ODC is in part responsible for the variations in the single and total polyamine contents. This enzyme affects mainly spermidine and putrescine levels, that are more involved in cell proliferation than spermine; the latter is implicated essentially in cell differentiation and neoplastic transformation, with different processes engaged in maintaining its critical levels (39).

Polyamines are also pivotal in the regulation of ion transport and the stabilization of several cellular components, such as cell membranes and chromatin structure. Therefore, a reduction of the levels of these polycations might induce destabilization of important cell structures, leading to loss of cell integrity and finally inducing cell death (39,40). It has been observed that the depletion of polyamines by treatment with the ODC inhibitor, difluoromethylornithine (DFMO), resulted in growth inhibition leading to a significant increase in apoptosis by altering both Bax and Bcl-2 expression (41,42).

Based on the above, the inhibitory effect on polyamine biosynthesis by VK1 could also be linked to the observed increase of apoptosis. VK1 concentrations ≥100 μM also induced apoptosis in both cell lines after 48 h of treatment, as indicated by the significant increase in the Bax/Bcl-2 ratio of mRNA levels. However, in HGC-27 no significant changes in Bax and Bcl-2 proteins occurred, probably due to the fact that VK1 exerted its effects on the modulation of gene expression without any consequence on the protein structure in this cell line. In the contrary, the increase in Bax/Bcl-2 ratio of mRNA levels in SW480 cells was caused by a significant increase in the pro-apoptotic Bax protein levels and a significant decrease in the anti-apoptotic Bcl-2 protein levels.

As regards the possible involvement of the MAPK pathway in the apoptotic process, treatment with VK1 showed a significant dose-dependent increase in ERK phosphorylation, with a peak increase after 48 h in SW480 cells, but not in HGC-27 ones. Of note, when SW480 cells were simultaneously co-treated with a MEK inhibitor and VK1, the induction of ERK phosphorylation was prevented and VK1-mediated induction of apoptosis was blocked, indicating the participation of the MAPK pathway in this process.

Although it is accepted that ERK pathway stimulates cell growth and protects cells from death (43), our findings agree with reports in literature proposing new interpretations of the ERK phosphorylation. Zhu *et al* (44) demonstrated a proapoptotic role of the ERK pathway in T-cells where the inhibition of ERK phosphorylation antagonized apoptosis. Similar results were obtained by Showalter *et al* (13) in a study performed on pancreatic cancer cell lines treated with either VK1 or VK2 administered at inhibitory doses. ERK phosphorylation was also induced by two VK analogs in different cancer cells and had a strong correlation with the growth-inhibitory effect (45). The increase in ERK phosphorylation could be closely linked to the decrease in polyamine contents observed after VK1 treatment. Previous studies have shown that the inhibitory effect on polyamine biosynthesis by DFMO treatment increases ERK phosphorylation and signaling through the MAPK pathway in human breast cancer cells (46).

Finally, a caspase-dependent induction in apoptosis induced by VK1 was not observed in the cell lines, since western blot analysis revealed no active products of caspase-3, an executioner caspase, and caspase-9, an initiator caspase. Even if caspases are established players of apoptosis in various models, increasing evidence demonstrates that apoptosis may be induced without their activation (47).

In conclusion, to the best of our knowledge, this is the first study aimed at comparing the effects of the naturally occurring VK1 on gastric and colorectal cancer cell growth and exploring its mechanisms of action. Our findings show an inhibition of cell proliferation and induction of apoptosis involving polyamine biosynthesis and MAPK pathway. Moreover, this evidence indicates a different proliferative behavior and a different response to VK1 by gastric and colon cancer cells, with the latter showing a more pronounced susceptibility to VK1 action. This cell line demonstrated growth ability closely linked to the high capacity of *de novo* polyamine biosynthesis and polyamine uptake (48). This evidence supports the hypothesis that VK1 may have different effects on different types of cell lines.

Although future studies are needed to better elucidate the exact mechanisms underlying VK1 anticancer properties and to check these effects also in *in vivo* models, our preliminary data imply potential for novel therapeutic strategies. VK1 is safe and without known toxicities in adult humans (49), so it could be effective in prevention and treatment of selected GI neoplasms. In particular, as hypothesized for other diet components, a combined chemopreventive and therapeutical intervention using protocols based on the use of this naturally substance, along with polyamine inhibitors and/or analogues would enhance VK1 properties. This could represent a suitable alternative option for improving the efficacy of chemotherapy and chemoprevention and the management of patients with cancers of the digestive tract.

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