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COMPARISON OF ANTITUMOR ACTIVITY OF VITAMINS K_1, K_2 AND K_3 ON HUMAN TUMOR CELLS BY TWO (MTT AND SRB) CELL VIABILITY ASSAYS

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Summary

Vitamin K (VK) congeners (VK_1, VK_2, and VK_3) have been used as antihemorrhagic agents, while VK_3 has also been found to inhibit growth in various rodent and human tumor cells. We have compared the antitumor activities of vitamin K_1, K_2, and K_3 against a panel of human cancer cell lines. For each test agent, a dose-response profile was generated by using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and an SRB (sulforhodamine B) assay. Both assays yielded similar results. The respective ID_{50} values of VK_3 in five hepatoma cell lines, HA59T, HA22T, PLC, HepG2, and Hep3B, of increasing differentiation state, were 42, 36, 28, 27, and 20 μM. For nasopharyngeal carcinoma (CG1), leukemia (U937), oral epidermoid carcinoma (KB), and breast carcinoma (BC-M1) cells, the ID_{50} values of VK_3 were 26, 15, 25, and 33 μM, respectively. For all the above cells, the ID_{50} values of VK_1 ranged from 6 to 9 mM, and the ID_{50} values of VK_2 ranged from 1 to 2 mM. Thus, the relative potencies of antitumor activity of VK_3 compared to VK_2 and to VK_1 are about 60- and 300-fold, respectively. These results support the preference for use of VK_3 over VK_1 and VK_2 in cancer therapy.

Vitamin K (VK) is a generic term for a homologous group of fat-soluble vitamins which promote blood clotting by increasing prothrombin synthesis in the liver. There are at least three such vitamins: vitamin K_1 (phytonadione), vitamin K_2 (menaquinone), and vitamin K_3 (menadione). Although all vitamin K naphthoquinones share certain biochemical properties and can serve as electron acceptors for mammalian redox enzymes, their differences in

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prosthetic group substitution impart them with important biochemical and physiologic differences.

VK_3 (menadione, 2-methyl-1,4-naphthoquinone) is a synthetic derivative of the naturally occurring VK_1 and VK_2. Compounds with a quinone structure have played a prominent role in cancer chemotherapy, particularly the anthracyclic quinones doxorubicin and daunorubicin. The antitumor activity of VK_3 was first evaluated by Mitchell et al. in 1965 [1] as a radiation sensitizer and later as a targeting agent for the delivery of radioisotope. Inhibition of tumor cell growth by VK_3 in a wide variety of rodent and human tumor cell types has been recognized more recently by other investigators [2] and our group [3-6]. Although, many possible mechanisms of action have been proposed, the exact mechanism by which VK_3 acts as an antineoplastic agent is still not well understood. Moreover, there is a paucity of information regarding VK congeners in the literature. VK_1 and VK_3 have been reported to be inhibitors of DNA synthesis in malignant cells [2].

Therefore, we have evaluated the anticancer activity of VK_1, VK_2, and VK_3 on various disease-oriented cancer cell lines by using 3-[4,5-Dimethyl-thiathiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [7-9] and sulforhodamine B (SRB) [10-11] assays. The cell lines include some that were established locally and some derived from the cancers which are most prevalent in Taiwan, such as hepatoma, cervical carcinoma, and nasopharyngeal carcinoma (NPC).

In addition, since decrease in cellular glutathione (GSH) pools as a consequence of VK_3 has been demonstrated using mouse leukemia [12,13], and human hepatoma cells [14]. We investigated whether direct chemical interaction between VK_3 and glutathione may play a role in VK_3-induced biochemical changes in cellular systems. Glutathione is the major non-protein thiol source in mammalian cells and tissues, cellular GSH is known to play an important role in the ultimate efficacy of several chemotherapeutic agents [15]. Therefore, the interactions of various concentrations of vitamin K congeners with GSH and the protective role of glutathione against VK_3-mediated toxicity in human tumor cells were also investigated.

Methods

Drugs, Chemicals VK_1, VK_2, SRB and MTT were obtained from Sigma Chemical Co. Menadione sodium bisulfite, trichloroacetic acid (TCA) and other reagents were purchased from Merck Chemical Co. Menadione sodium bisulfite was dissolved in phosphate-buffered saline (PBS). VK_1 and VK_2 were dissolved in ethanol and aliquots of 0.1 M solutions were stored at -20°C. Dilutions for use in experiments were prepared in complete medium immediately before use.

Cell Lines The human tumor cell lines used were hepatoma, lung carcinoma, nasopharyngeal carcinoma (NPC), breast carcinoma, oral epidermoid carcinoma, leukemia, stomach carcinoma, lymphoma, and colon carcinoma. NPC (CG1) cells were provided by Dr. Y.S. Chang of Chang-Gung Medical Center, Lin Kou, Taiwan. All cells were grown in DMEM (high glucose, Gibco Lab, NY) medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM non-essential amino acids and 100 U/100 $\mu g/ml$ of penicillin/streptomycin in a 5 % CO_2-95 % air incubator at 37 °C. Established

adherent monolayer cells approaching 80% confluency were harvested with trypsin/EDTA (Gibco Lab, NY).

MTT-Microculture Tetrazolium Assay The methodology described below represents a modified MTT colorimetric assay reported by Alley MC et al [8]. In brief, cells were harvested from cultures maintained in the exponential phase, counted by trypan blue exclusion, and dispensed into 96-well culture plates (Falcon Plastics) in 100 μl volumes. Following overnight incubation at 37 °C, 100 μl of culture medium containing drug was dispensed into appropriate wells in triplicate. Culture plates were then incubated for 2 days prior to the addition of a tetrazolium reagent. All but 10-20 μl of culture medium supernatant was removed from wells by slow aspiration and replaced with 150 μl of DMSO. After 10 min shaking to solubilize formazan, the absorbance at 540 nm of each well was measured using a microtiter plate reader (Model EL340, Bio-Tek Instruments, Inc.) [7-9].

SRB-Microculture Assay The SRB assay was established by the U.S. National Cancer Institute for large-scale screening of antitumor drugs in micro-plates [10]. Cell cultures were fixed with TCA before washing, incubated at 4 °C for 1 hr, and then washed five times with tap water by an automated washer (Model EL403, Bio-Tek Instruments, Inc.) to remove TCA, growth medium, and low-molecular-weight metabolites. Plates were air dried and then stored until use. TCA-fixed cells were stained for 30 min with 0.4% (wt/vol) SRB dissolved in 1 % acetic acid. At the end of the staining period, SRB was removed and cultures were quickly rinsed five times with 1 % acetic acid to remove unbound dye. Then the cultures were air dried until no conspicuous moisture was visible. Bound dye was solubilized with 10 mM unbuffered Tris base (pH 10.5). Plates were shaken for 5 min. The absorbance at 515 nm of each well was measured using a microtiter plate reader (Model EL340, Bio-Tek Instruments, Inc.) [10, 11].

Fluorometric Assay for Glutathione Sample solutions were buffered by the addition of 0.5 ml of 0.1 M sodium phosphate buffer, pH 8.0, o-phthalaldehyde (0.1 ml) was added with thorough mixing. After a 15-20 min reaction period at room temperature, the mixture was transferred to a quartz curvet and the fluorescence at 420 nm resulting from excitation at 350 nm was determined.

Data Management The data were stored and analyzed through the use of Kineticalc 2.03 and ΣPlot 4.0 software. Cell growth and growth inhibition are expressed in terms of mean (±1 SD %) percentage of control absorbance following subtraction of mean background absorbance.

Results

Comparison of Antitumor Activity of Vitamins K_1, K_2 and K_3 in Human Tumor Cells by Two (MTT and SRB) Cell Viability Assays

We have compared the antitumor activities of vitamins K_1, and K_2 with that of VK_3 in various disease-oriented cancer cell lines. Both SRB and MTT assays yielded similar results (see TABLES I and II). The respective ID_{50} values of VK_3 in hepatoma cell lines HA59T, HA22T, PLC, HepG2, and Hep3B, of increasing differentiation state, were 42, 36, 28, 27, and 20 μM. For cervical cancer (HeLa, SiHa,

and TSGH8302) (See F.Y.-H. WU, T.-P. SUN, W.-C. CHANG, and C.-C. JUAN, Cancer Res., Submitted), NPC (CG1), leukemia (U937), oral epidermoid carcinoma (KB), and breast carcinoma (BC-M1) cells, the ID_{50} values of VK_3 were 50, 37, 37, 26, 15, 25, and 33 μM, respectively. For all the above cells, the ID_{50} values of VK_1 ranged from 6 to 9 and the ID_{50} values of VK_2 ranged from 1 to 2 mM. Thus, the relative potencies of antitumor activity of VK_3 compared to VK_2 and to VK_1 is about 60- and 300-fold, respectively.

Interaction of Vitamin K Congeners with Glutathione The fate of various concentrations of vitamin K congeners when mixed with GSH is shown in FIG 1. The doses required for 50% removal of GSH by VK_3, VK_2, and VK_1 were 50 μM, 4.9 mM, and 18 mM, respectively.

Modulation of Cytotoxicity of VK_3 by Glutathione As shown in TABLE III, VK_3 toxicity to CG1, HA22T, and HepG2 cells was completely prevented by a high level (1 mM) of GSH.

TABLE I

ID_{50} Values of Cell Lines Treated with Vitamin K Congeners as Determined by MTT Assay

Cell Lines	ID_{50}[a]		
	VK_1 (mM)	VK_2 (mM)	VK_3 (μM)
Liver cancer			
HA59T	>10.0	0.8	45
HA22T	8.5	1.0	33
PLC	>10.0	0.8	26
HepG2	5.0	1.0	23
Hep3B	5.2	0.8	18
Colon cancer			
COLO 320DM	>10.0	3.0	10
Leukemia			
U937	>5.0	>2.0	15
Lung cancer			
MRC-5	>10.0	0.8	18
Stomach cancer			
KATO-III	9.0	1.1	22
Lymphocyte cancer			
Raji	1.6	0.2	13
Nasopharynx cancer			
CG1	>10.0	>2.0	28
Breast cancer			
BC-M1	>10.0	>2.0	35
Oral epidermoid cancer			
KB	>10.0	>2.0	20

[a]Data represent the means of triplicate cultures.

Discussion

This is the first and the most comprehensive comparison of the antitumor activities of vitamins K_1, K_2 and K_3 based on MTT and SRB assays using more than 15 cell lines representing 10 diseases. Some cell lines used were derived from the cancers which are most prevalent in oriental populations such as hepatoma, cervical cancer and NPC. These cell lines are not included in the cell line panel used by the National Cancer Institute [11] and other studies [1-3, 12-14]. The information on the relative antitumor potencies of VK congeners against various human cancer cell lines was scarce. Furthermore, these relative potencies have never been determined systematically. In our study and on the basis of the ID_{50} values determined, VK_3 was found to be the most potent anticancer drug among VK congeners. Moreover, VK_3 had a wide spectrum of cytotoxicity against cancer cells from liver, cervix, nasopharynx, colon, leukemia, lung, stomach, lymphocyte, and breast cancers.

TABLE II

ID_{50} Values of Cell Lines Treated with Vitamin K Congeners as Determined by **SRB** Assay

	ID_{50}[a]		
Cell Lines	**VK_1 (mM)**	**VK_2 (mM)**	**VK_3 (μM)**
Liver cancer			
HA59T	>10.0	0.4	42
HA22T	9.0	1.0	36
PLC	>10.0	0.8	28
HepG2	4.9	0.8	27
Hep3B	5.0	0.6	20
Colon cancer			
COLO 320DM	10.0	5.0	8
Leukemia			
U937	>5.0	>2.0	15
Lung cancer			
MRC-5	>10.0	0.7	16
Stomach cancer			
KATO-III	10.0	1.0	20
Lymphocyte cancer			
Raji	1.5	0.1	9
Nasopharynx cancer			
CG1	>10.0	>2.0	26
Breast cancer			
BC-M1	>10.0	>2.0	33
Oral epidermoid cancer			
KB	>10.0	>2.0	22

[a] Data represent the means of triplicate cultures.

TABLE III

Effect of Glutathione on the Viability of CG1, HepG2, and HA22T Cells Treated with VK_3[a]

Cell Lines	Without 1 mM GSH		With 1 mM GSH	
	VK_3 concentration (μM)			
	40	100	40	100
CG1	24.5 ± 4.9[b]	16.7 ± 4.2	113.0 ± 13.8	106.7 ± 14.2
HA22T	38.0 ± 8.6	22.7 ± 7.3	85.4 ± 8.7	99.8 ± 6.9
HepG2	24.7 ± 5.6	16.6 ± 3.5	85.3 ± 9.3	96.5 ± 11.9

[a] Cells were seeded at 5 x 10^5 cells/dish. After 24 hr for cell attachment, cells were incubated with 40 and 100 μM VK_3 in the absence or presence of GSH, then allowed to recover for 24 hr. Cell viability was determined by trypan blue exclusion.

[b] The values are all listed as percentages of viabilities of control untreated cells, and each value represents the average from three dishes.

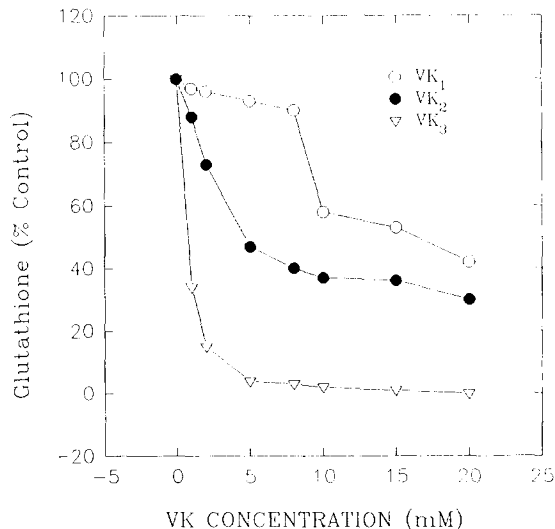

FIG. 1

Interaction of VK congeners with glutathione (GSH). VK_1, VK_2 or VK_3 (100 μM - 20 mM) were incubated with 100 μM GSH at 37 °C for 1 hr. Data are expressed as percentages of the starting GSH concentration.

In this study, we further confirmed that the SRB and the MTT assays yielded similar results [11]. The MTT assay relies on the ability of viable cells to reduce a tetrazolium dye to formazan. While such an assay can be very simply performed, it is based on the assumption that dead cells or their products do not reduce tetrazolium. On the other hand, the SRB assay measures the whole-culture protein content. To determine cell growth or viability, one assumes that dead cells either lyse or are removed during the procedure, or otherwise do not contribute to the colorimetric end point. The SRB assay appears to offer several advantages over the MTT assay for large-scale drug screening being simpler, faster, and more sensitive than the MTT assay. We have established a semi-automated system and disease-oriented cell line panel for future large-scale screening of antitumor drugs in Taiwan.

It is likely that the mechanism of VK_3 cytotoxicity is more complex and broader than those of VK_1 and VK_2. Akman and co-workers [12] demonstrated that reduced nicotinamide adenine dinucleotide phosphate (NADPH) was depleted by VK_1 or VK_3. In contrast, VK_3 depleted the GSH pool to a significantly greater degree than do equitoxic concentrations of VK_1 or other analogues. These results seem to suggest that GSH depletion is an important but not a general mechanism of toxicity for all antitumor quinones. However, our results indicated that there was a good correlation between growth inhibition and GSH depletion among vitamin K congeners. The difference in cytotoxicity of vitamin K congeners in cancer cell lines may be related to the relative potency of congeners in depleting GSH.

The protective effect of GSH against VK_3-induced growth inhibition has previously been demonstrated in mouse L1210 leukemia cells [12], and human hepatoma cells [14]. In this study, we observed complete protection from VK_3 toxicity in CG1, HA22T, and HepG2 cells by a high level (1 mM) of GSH. Similar results were also obtained by using other tumor cell lines (Data not shown). However, the efficacy of protection decreased either when GSH dose was < 1 mM and VK_3 was kept 100 μM or when VK_3 dose was >100 μM and GSH was kept 1 mM, and there was no protective effect by the addition of 1 mM GSH after cells had been exposed to 100 μM VK_3 for 1 hr (Data not shown). These results suggest that cells are affected by VK_3 only when the oxidative stress of VK_3 surpasses the reducing ability of GSH, and that VK_3-induced cellular or genetic damage occurs within 1 hr. Since the GSH pool is an important determinant of resistance to some chemotherapeutic agents, VK_3, among the most potent GSH-depleting agents, confers a therapeutic advantage beyond its cytotoxicity in clinical chemotherapy, especially in the combination treatment with other antitumor agents.

In summary, the relative antitumor potencies of 1/60/300 for $VK_1/VK_2/VK_3$ were deduced from this study. These results support the preference for VK_3 over VK_1 and VK_2 in cancer therapy. VK_3 cytotoxicity observed in our _in vitro_ studies is supported by the information obtained from an _in vivo_ tumor-bearing animal model system [4, 16]. Furthermore, in preliminary phase I clinical trial, when VK_3 at doses of 40-3200 mg/m^2 per course administered i.v. over 1-4 days, no major toxic effects were observed [17]. In one pharmacokinetic study [18], plasma VK_3 concentration up to 7.4 μM was achieved during the infusion in 3 patients receiving 1360 mg/m^2. The levels of VK_3 achieved safely in the clinical

trials are similar to those associated with in vitro cytotoxicity against human cell lines in our studies. These provide useful information for future clinical trials of VK_3 in the treatment of common cancers in Taiwan. The mechanisms of VK_3 action against various human cancer cells have been the focus of our research. VK_3 was found to induce cell cycle arrest or progression delay at the S/G_2 phase [5] which was associated with the decreased activity and hyperphosphorylation of $p34^{cdc2}$ kinase [19]. VK_3 also induces apoptosis [6] which includes the alteration in expression of c-myc and c-fos proto-oncogenes [20].

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