

The Anticancer Effects of Vitamin K

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Abstract

Vitamin K, an essential nutrient often associated with the clotting cascade, has been the focus of considerable research demonstrating an anticancer potential. Much of this research has focused on vitamin K3, although vitamins K2 and K1 have also been shown to have anticancer effects. Early studies of vitamin K3 employed an oxidative model to explain the anticancer effects seen in both *in vitro* and *in vivo* studies; however, this model does not adequately address the action of vitamins K1 and K2. Recent research has demonstrated the anticancer action of vitamin K may act at the level of tyrosine kinases and phosphatases, modulating various transcription factors such as Myc and Fos. Tyrosine kinases associated with cyclins have also been shown to be affected by vitamin K, which can lead to cell cycle arrest and cell death. (*Altern Med Rev* 2003;8(3):303-318)

Introduction

After earlier research, a purified form of vitamin K, phylloquinone, was isolated from plants in 1939 and used to treat a nutritional deficiency characterized by decreased prothrombin levels. Henrick Dam received the Nobel Prize in 1943 for his discovery of vitamin K (the "koagulations" vitamin). The role of vitamin K as a cofactor in normal blood coagulation stems from the post-translational modification of a number of plasma proteins such as factors II (prothrombin), VII, IX, X, as well as proteins C, S, and Z. Vita-

min K, in its reduced hydroquinone form, acts as a cofactor in the enzymatic carboxylation by gamma-glutamyl-carboxylase of glutamic acid residues forming gamma-carboxyglutamic acid in plasma proteins.¹ In the process of carboxylation, vitamin K epoxides (2,3 epoxides) are formed, which are reduced back to vitamin K by thiols and epoxide reductases. Thus, vitamin K cycles from an epoxide to a quinone and back to the hydroquinone for another gamma-carboxylation reaction. The drug Coumadin® (warfarin) inhibits vitamin K epoxide reductase, interfering with the reduction of the epoxide and halting the cycling back to the hydroquinone intermediate, thereby interrupting the activation of blood coagulation factors.²

Although vitamin K is usually identified as a critical factor in blood coagulation, recent research has found that vitamin K is also a cofactor in bone metabolism.³⁻⁹ Inhibition of cancerous cell growth *in vivo* and *in vitro* by vitamin K has also been observed.¹⁰⁻¹⁷ Examination of this latter phenomenon, its mechanism and related concepts, comprises the balance of this paper.

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Vitamin K-Dependent Receptors

The role of vitamin K as a cofactor in the carboxylation of gamma-carboxyglutamyl protein residues has expanded to include a new class of vitamin K-dependent receptor:ligand systems critical to aspects of cellular metabolism. Several vitamin K-dependent proteins have been identified as specific ligands for receptor tyrosine kinases (RTKs) that are important in a number of cell signaling processes such as cellular survival, transformation, and replication.¹⁸

Growth-arrest-specific gene-6 (Gas6) is an example of a vitamin K-dependent protein ligand that increases in growth-arrested cells.¹⁹ Protein S, like Gas6, also acts as an RTK ligand. After translation Gas6 protein is carboxylated via a vitamin K-dependent process in the endoplasmic reticulum. This post-translational modification enables Gas6 to bind to a number of receptor protein tyrosine kinases such as Axl (also designated as Ufo or Ark), Sky (also designated Dtk, Tyro3, Rse, Brt, Etk2, or Tif), and Mer (also Eyk or Tyro12) that make up a new subfamily of vitamin K-dependent receptor tyrosine kinases.^{20,21} Various names have been assigned to the same receptors because they were described by different investigators using a number of cell lines. Recently, many have been found to be identical. A major function of Gas6-Ark signaling involves increased cell survival under conditions that do not allow cell proliferation.²² Although the exact function of Gas6 protein is not yet fully defined, it is believed it may act as a physiological anti-inflammatory.²³ It is also part of a mechanism for clearing away apoptotic and dying cells by helping phagocytic cells to recognize phosphatidylserine-expressing cells.²⁴

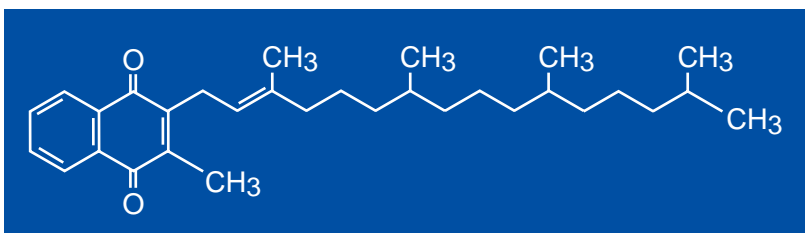
This newly discovered subfamily of receptor tyrosine kinases has also been associated with cell growth regulation and tumorigenesis. Discussions of vitamin K-dependent proteins related to lymphoid malignancy,²⁵ lung

malignancy,²⁰ and multiple myeloma²⁶ have been published. While they were not related to possible treatment of malignancy with vitamin K derivatives, they are mentioned here for completeness.

Vitamin K Structure

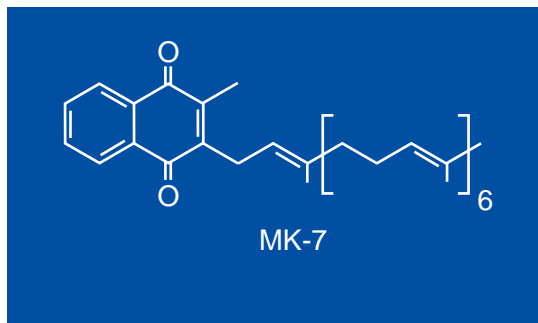
Vitamin K is a family of structurally similar fat-soluble 2-methyl-1,4-naphthoquinones, including phylloquinone (K1), menaquinones (K2), and menadione (K3). 1,4-Naphthoquinones form a family of compounds characterized by a naphthalene ring containing two carbonyl moieties at positions 1 and 4, which in the case of vitamin K is substituted at positions 2 and 3 (Figures 1-3). All members of the vitamin K family possess the identical naphthoquinone skeleton with various side chains that distinguish them. The best-known member of the vitamin K family is phylloquinone, also known as phytonadione or menaphthone, so named because of its intimate relationship with photosynthesis in plant leaves. Phylloquinone is found in many higher plants as well as algae, with the highest concentrations found in green leafy vegetables.²⁷

Figure 1. Phylloquinone (Vitamin K1)



Menaquinones (K2) also occur naturally, but are not produced by plants; rather, they are produced by a vast array of bacteria. Menaquinones were originally isolated from putrefied fishmeal as a product of microbial synthesis.²⁸ Recent studies have discovered menaquinones can actually be produced by animals and probably humans from the conversion of other

**Figure 2. Menaquinones
(Vitamin K2)**



forms of vitamin K.^{29,30} The most common form of vitamin K in animals is menaquinone 4 (MK-4), produced by intestinal bacteria from exogenous naphthoquinones and transformed endogenously in our own cells.¹ Vitamins K1 and K2 differ only in the prosthetic group at position 3 (Figures 1 and 2). Vitamin K1 possesses a phytyl group (partially saturated poly-isoprenoid group) at position 3, while K2 possesses a repeating unsaturated trans-poly-isoprenyl group. The IUPAC-IUB Commission on Biochemical Nomenclature abbreviates phyloquinone (K1) as “K” while menaquinone (K2) is abbreviated as “MK-n.” The “n” signifies the number of unsaturated isoprene units that compose the side chain at the 3-position. The side chain of MK-n can vary in length from C₅ (n=1) to C₆₅ (n=13). For example, menaquinone 7 (MK-7) could also be written as K₂₃₅. MK-7 has six isoprene units plus the first saturated group beginning at position 3, equaling seven (Figure 2). It is believed that bacteria produce a series of menaquinones with 85-95 percent having an n of 7, 8, or 9.³¹

Menadione (K3) is not considered a natural vitamin K, but rather a synthetic analogue that acts as a provitamin. It possesses a much simpler structure, with no aliphatic chain prosthetic group at position 3 (Figure 3).

Although menadione is considered a synthetic analogue, Billeter et al found that phyloquinone can be cleaved to form menadione by bacteria in the intestine.^{29,32} After absorption, menadione is thought to become alkylated into biologically active isoprenylated menaquinones. However, K3 cannot exert all the functions of natural vitamin K, which is ascribed to limited transformation into the fat-soluble vitamin forms.^{31,33}

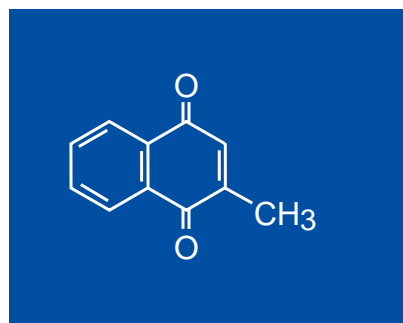
Cancer Research on Vitamin K *Menadione (Vitamin K3)*

The antitumor action of vitamin K has been under investigation since 1947.^{34,35} Menadione (150-200 mg/day IV), as a radiosensitizing agent, was discovered to increase survival time (5.42 months with menadione and radiation versus 3.77 months with radiation alone) in inoperable bronchial carcinoma patients.³⁶ Pretreatment of mice with transplanted mouse liver tumors by oral or intraperitoneal injection of vitamins K3 and C greatly potentiated the action of radiation (20-40 Gy dosages) compared to controls.³⁷ In rats, menadione was active against adriamycin-resistant leukemia cells.³⁸ Hepatoma-bearing rats receiving intraperitoneal injections of menadione (10

mg/2mL weekly for four weeks) demonstrated an increased survival rate of 60 days compared to 17 days for controls (five of 16 lived longer than controls).¹⁵ The anticancer activity of menadione has also been demonstrated in a number of *in vitro* studies using both rodent^{10,39-42} and human cancer cell lines.^{11,12,43-45} Menadione was effective against multidrug-resistant leukemia cell lines and parental leukemia cell lines.⁴³

Both *in vivo* and *in vitro* studies showed a synergistic effect when menadione was combined with conventional chemotherapeutic agents. Combining menadione (5x10⁻⁷ to 1x10⁻⁶ M) and 5-fluorouracil (5-FU) significantly enhanced the action

**Figure 3. Menadione
(Vitamin K3)**



against hepatoma cells.⁴² Menadione was found synergistic with 5-FU, bleomycin, cisplatin, and dacarbazine in human oral epidermoid carcinoma (KB) cell culture. Menadione in KB cell cultures also demonstrated an additive effect when combined with 10 other chemotherapeutic agents (mercaptopurine, cytarabine, hydroxyurea, VP-16, vincristine, doxorubicin, mitoxanthine, mitomycin C, actinomycin D, and thiotepa).⁴³ The synergistic action between K3 and doxorubicin, 5-FU, and vinblastine was also demonstrated in nasopharyngeal carcinoma cells.⁴⁶ The combination of mitomycin C and menadione led to a 10- to 50-fold reduction in the levels of mitomycin C required for direct cytotoxicity.⁴⁷ Pretreatment with menadione before doxorubicin or mitomycin increased cytotoxicity to MCF-7 breast cancer cells.⁴⁸

The encouraging results of this earlier research have led to a number of *in vivo* trials. Studies in rats determined that menadione at blood levels of $\leq 1 \mu\text{M}$ showed synergy with methotrexate without an increase in toxicity. A minimum threshold for synergy was determined to be greater than 200 mg/kg/day and not more than 250 mg/kg/day of vitamin K3. The combination of methotrexate (0.75 mg/kg/day) with menadione (250 mg/kg/day) resulted in a 99-percent inhibition of tumor growth, while decreasing the dosage of K3 to 225 mg/kg/day led to an 84-percent inhibition.¹⁶

Human studies to date investigating the effects of menadione and mitomycin C have shown mixed results. A phase I trial determined a maximum tolerated menadione dose of 2.5 g/m² to be a 48-hour intravenous infusion followed by mitomycin C (15 mg/m²) every four weeks with no resulting hemolysis.⁴⁷ This trial has been followed up by two phase II trials of menadione in combination with mitomycin C. The first trial involved 23 advanced lung cancer patients with an overall median survival of 5.5 months. Two patients had objective response lasting 3.5 to 13 months. Twenty-six percent had some tumor regression. Thirty percent were complicated by hematologic toxicity (hemolytic uremic syndrome, hemolytic anemia, or hematological parameters that did not return to normal levels in two weeks).⁴⁹ In the second trial, 43 gastrointestinal cancer patients showed no objective response to the therapy.⁴⁸

Vitamin K3 in combination with vitamin C without the concomitant use of chemotherapy and radiation has shown anticancer effects both *in vitro* and *in vivo*. Menadione in combination with vitamin C (ratio of 100:1, vitamin C: vitamin K3) achieved cytotoxic dosages 10-50 times lower than when singly administered. Menadione at 13.8 $\mu\text{g/mL}$ produced a 50-percent inhibition of breast cancer (MCF-7) cell growth. When combined with vitamin C (99.01 $\mu\text{g/mL}$ or 500 $\mu\text{mol/L}$), K3 (1.38 $\mu\text{g/mL}$) increased inhibition by 74 percent. Increasing the concentrations of vitamins C and K (10⁴ $\mu\text{mol/L}$ and 10⁵ nmol/L, respectively) produced a 93-percent inhibition. Using KB cells, a 50-percent inhibition of growth was seen with K3 (13.8 $\mu\text{g/mL}$). When KB cells were subjected to vitamin C at 10³ mmol/L or K3 at 10⁴ nmol/L separately, no *in vitro* cytotoxic effect resulted. When combined at the same concentrations, a synergistic inhibition of 100 percent was achieved. Human endometrial adenocarcinoma cells subjected to similar concentrations of the two vitamins resulted in similar inhibition of growth.¹²

The combination of vitamins C and K3 using the ratio of 100:1, vitamin C: vitamin K3, was also found to inhibit a number of urological cancer cell lines, with a 4- to 61-fold potentiation of cytotoxic activity. The 50-percent cytotoxic dose (CD₅₀) values were categorized into two sensitivity groups. The first group included cell lines of clear cell kidney carcinoma grade I, transitional-cell bladder carcinomas grade III/IV and IV/IV, and human squamous cell bladder carcinoma grade III/IV. A 10- to 21-fold decrease in CD₅₀ with a vitamin C/vitamin K3 ratio of 89 μM /0.9 μM was demonstrated, in contrast to the individual vitamin cytotoxicities. The second group of cell lines included prostate carcinoma Gleason grade IV, transitional-cell papilloma of the bladder grade II/IV and III/IV, and embryonal carcinoma of the testis grade IV. A 7- to 22-fold decrease in CD₅₀ values was demonstrated with a vitamin C/vitamin K3 ratio of 212 μM /2.13 μM .⁵⁰

Similar results have been observed in the androgen-independent human prostate cell line DU145 and bladder carcinoma cell line T24 with

a CD_{50} value of 212 μM for vitamin C and 2.13 μM for vitamin K3 in T24 cells.⁵¹ Co-administration of vitamins K3 and C (100:1) was found to enhance the cytotoxic antitumor effect in human prostate carcinoma cell lines by 5- to 20-fold over either single agent.⁵⁰ In another study on prostate cancer, human prostate tumors implanted in mice were treated with 100:1 vitamin C to K3. This combination restored the essentially absent DNase activity essential for apoptosis.⁵²

The *in vitro* synergistic cytotoxic effects of vitamins C and K3 were shown to be more sensitive in cancer cells such as oral squamous carcinoma and human promyelocytic leukemia when compared to normal cells such as fibroblasts and pulp cells.⁵³ The cytotoxic action of this combination of vitamins C and K3 is characterized by a cell death that is morphologically distinct from apoptosis and necrosis, referred to as autoschizis. During the process of autoschizis, cytoplasm is extruded leaving an intact nucleus.⁵⁴

A number of *in vivo* studies demonstrate the synergistic combination of vitamins K3 and C in cancer treatment. Mice exhibiting ascites and liver tumors resistant to the vincristine alkaloid Oncovin® were found to be sensitive to Oncovin when pretreated with the combination of vitamins K and C, without any resultant organ toxicity.⁵⁵ Nude mice with Du145 human prostate cancer were given vitamins K3 and C, either orally or by injection, and were found to have a 25-percent longer mean survival time than controls, some outliving controls.⁵⁶

Phylloquinone (K1)

Although most of the anticancer research of vitamin K has focused on K3, there have been a number of studies demonstrating the anticancer effects of K1. Phylloquinone has been found to exhibit anticancer activity in a number of cell lines (liver, colon, lung, stomach, nasopharynx, breast, oral epidermoid cancer, and leukemia), with a 50-percent cell growth inhibitory dosage (ID_{50}) in the range of 2-10 mM.⁶ Other cell lines required less concentrated K1. Neuroblastoma cells (NBP2) exhibited an ID_{50} with 50 μM of K1 in serum.¹⁰ Leukemia cells (L1210) exhibited a 50-percent

inhibitory concentration (IC_{50} equivalent to ID_{50}) with 46 $\mu\text{g}/\text{mL}$ K1, while 70-percent inhibition resulted from 75 $\mu\text{g}/\text{mL}$.³⁹ The human hepatoma cell line Hep3B demonstrated an IC_{50} for K1 at 660 μM .⁵⁷ Another study found that concentrations of 100-300 $\mu\text{g}/\text{mL}$ arrested cell growth.⁵⁸ Although a number of cell lines required supraphysiologic concentrations of K1 for an ID_{50} , others responded to physiologic concentrations.

A number of human trials demonstrate the anticancer effects of vitamin K1. Thirty patients with hepatocellular carcinoma received oral K1 (40 mg daily). Seven patients had a partial response and six patients had disease stabilization (four greater than one year; two greater than two years). In seven patients, liver function improved with no resulting coagulopathy and in 15 patients the under-carboxylated prothrombin normalized.⁵⁸ In a phase I study of 40 hepatocellular carcinoma patients receiving oral K1 (40 mg/day), all patients evaluated had a 20-percent tumor response rate, with five patients living greater than one year on treatment.⁵⁹ Several phase II human trials using K1 in the treatment of hepatoma patients have resulted in positive results. A phase I/II trial of K1 with hepatoma patients resulted in decreased cancer growth.⁶⁰ A phase II study of 14 hepatocellular carcinoma patients (who had World Health Organization performance status of 3 or better with a prognosis of more than three months and no concomitant use of other systemic treatments for their cancer) employed oral K1 (Konakion® 20 mg) twice daily until disease progressed. Patients were evaluated every four weeks for toxicity and every eight weeks for response over a six-month period. Nine patients were evaluable for response (median age: 64 (54-80)); two had previous therapy with Tamoxifen, two had surgery, and one had chemoembolization. Four patients of the nine were reported to have stable disease, while five progressed. No toxicity was found in any of the participants.⁶¹

Menaquinones (K2)

Both *in vitro* and *in vivo* studies have shown that vitamin K2 also exhibits anticancer effects. A number of cancer cell lines were screened (including liver, colon, leukemia, lung, stomach, lymphocyte, nasopharynx, breast, and oral epidermoid) in an identical manner as K1, for inhibition by vitamin K2. Vitamin K2 was found to have an ID_{50} value from 0.8-2 mM, which, although lower than K1, is still much higher than inhibitory levels of K3 (18-45 μ M).⁶

Other *in vitro* studies have found lower concentrations of menaquinones are effective anticancer agents for a number of cancer cell types. The HOS TE85 human osteosarcoma cell line and the MC3T3-E1 mouse osteoblastic cell line were cultured for three days in a medium containing various concentrations of MK-4. The proliferation of HOS cells was suppressed by vitamin K2 in a dose-dependent manner up to 56 percent of control by 10^{-7} M of K2, and that of MC3T3-E1 cells was suppressed to 84 percent of control by 10^{-6} M of vitamin K2.⁶² MK-3 had an ID_{50} of 112 μ M (112×10^{-6} M) for the human hepatoma cell line Hep3B.⁶³

Vitamin K2 induced growth inhibition via cell cycle arrest and apoptosis in a dose dependent manner for glioma cells in both rat (C6) and human cell types (RBR17T, T98G).⁶⁴ Incubation with 3 μ M of MK-4 for 72 hours decreased cultured leukemic blast cells from 27.6 percent to 17.7 percent. Increasing the concentration of MK-4 to 10 μ M decreased blast cell numbers to 3.9 percent.⁶⁵ K2 was able to induce cell cycle arrest in the G_0G_1 transition as well as induce apoptosis in a dose-dependent manner in glioma, hepatoma, and leukemia cell lines.^{64,66,67} Leukemia cell lines resistant to apoptosis still demonstrated induction of differentiation.⁶⁷

Myeloblastic (ML1) and promyelocytic (HL60) cell lines cultured with 1 μ M of K2 showed an 84-percent differentiation induction as measured by nitroterazolium blue staining (NBT), while K1 showed no differentiation effect. Other differentiation-inducing agents such as retinoic acid, interferon-gamma, and camptothecin were found to have a synergistic effect when combined

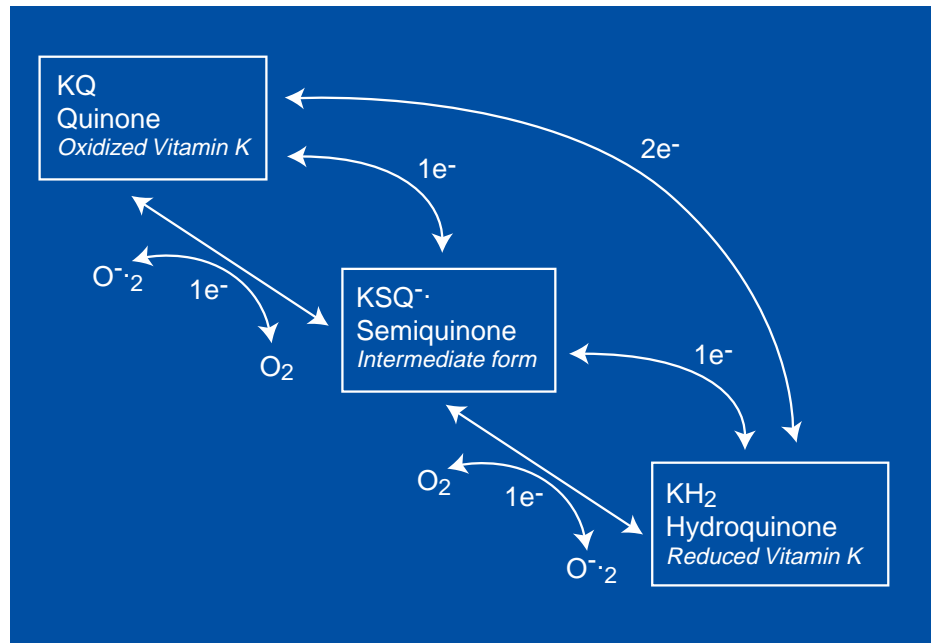
with K2.^{68,69} Further studies with isolated leukemia cells (post-myelodysplastic syndrome (MDS) and acute myelocytic leukemia) determined that K2 at 10 μ M was able to selectively induce apoptosis in the leukemia cells in 48 hours.⁶⁹ Three naturally occurring vitamin K2 analogues, MK-3, MK-4, and MK-5, were tested with leukemic blast cells. One of the more potent K2 analogues tested, MK-4 (a menaquinone with a geranylgeranyl (four isoprenoid units) prosthetic group at position 3), was found to induce apoptosis in 90 percent of leukemic blast cells. Normal bone marrow cells were also tested with the same concentration of the K2 analogues for 72 hours. The cytotoxicity to leukemia cells was much more pronounced, while the K2 analogues had "almost no effect" on normal bone marrow cells.⁶⁹ The selective cytotoxic effect of these three K2 analogues on immature transformed blasts was confirmed using a more sensitive antibody APO2.7 technique.⁷⁰

A number of striking case studies support the use of K2 as an anticancer agent. An 80-year-old woman with MDS received an oral dose of 45 mg/day of K2. After 14 months of treatment her pancytopenia improved and transfusions were no longer needed.⁷¹ A 72-year-old woman diagnosed with acute promyelocytic leukemia achieved remission when given all-trans-retinoic acid (60 mg/day), encitabine (200 mg/day), and daunorubicin (40 mg/day) for one week. Relapse occurred eight months later at which time 20 mg/day of vitamin K2 as MK-4 (Menatetrenone®, route of dose unspecified but assumed to be oral) in conjunction with the previous protocol resulted in the complete disappearance of promyelocytes after two months. Analysis of bone marrow confirmed complete cytogenetic remission.⁷² A 65-year-old man with MDS who had progressed to acute myeloid leukemia was treated orally with 90 mg/day of MK-4. Within six weeks he experienced a significant decrease in blast count from 34 to eight percent and increase in platelet count from $31 \times 10^9/L$ to $133 \times 10^9/L$. At 10 months the dosage was reduced to 45 mg/day with an absence of side effects and continuing good performance without myeloablative therapy.⁶⁵

These encouraging results from K2 therapy have lead to a multi-center pilot study in Japan of MDS and post-MDS acute myeloid leukemia (post-MDS AML) treatment with K2 (MK-4).⁷³ In the 11 independent institutes, 47 patients received treatment with MK-4. Of 47 patients, 15 had refractory anemia; six had refractory anemia with excessive blast; 11 had refractory anemia with excess of blast and in transformation; three had chronic myelomonocytic leukemia; and 12 had post-MDS AML. MK-4 was effective in reducing blast cell numbers in bone marrow and/or peripheral blood in 71.4 percent (10/14) of those receiving other medications concomitantly. Patients with refractory anemia with excess of blast in transformation, and those with post-MDS AML who used oral vitamin MK-4 without other medications demonstrated 44.4 percent (4/9) hematological improvement. MK-4 dosage ranged from 20-135 mg/day orally or 10-50 mg/day intravenously, with 83 percent receiving an oral dose of 45 mg/day.⁷³

A recent Japanese trial enlisted 121 patients with hepatocellular cancer undergoing conventional therapy consisting of percutaneous tumor ablation and/or transcatheter arterial embolization. Patients were given 45 mg/day oral vitamin K2, which resulted in a significant improvement in survival. Portal vein invasion after 12 months was two percent in the treatment group compared to 23 percent in the control group. At two years, 23 percent of the treatment group compared to 47 percent in the control group were found to have invasion into the portal vein.⁷⁴

Figure 4. Redox Cycling of Vitamin K



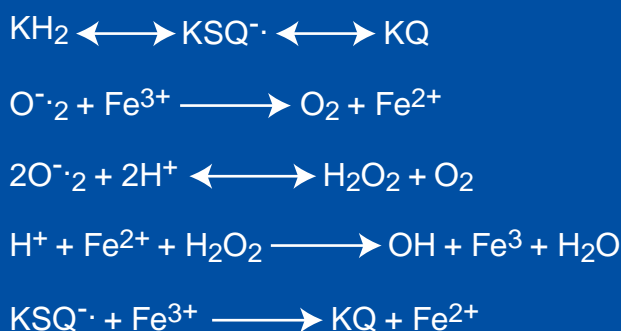
Theories of Action

The growth inhibitory and cytotoxic effects of vitamin K have been demonstrated both *in vivo* and *in vitro*, yet the mechanisms of action remain unclear. A number of mechanisms have been proposed and the dominant model has focused on the oxidative capacity of vitamin K analogues such as menadione. More sophisticated theories that do not fit into the oxidative model have been proposed to explain puzzling aspects of the anticancer effects of vitamin K1 and K2 versus K3, such as the induction of apoptosis, differentiation, and cell cycle inhibition.

The Oxidative Model

Historically the effectiveness of menadione against cancer was believed to be due to oxidative stress via redox-cycling of the quinone to produce reactive oxygen species (ROS) such as the hydroxyl radical, superoxide radical, and hydrogen peroxide.⁷⁵ The increased redox-cycling of menadione and the production of ROS surpasses the oxidative capacity of the cell, resulting in cell death. Quinones can undergo either one-electron reduction, producing semiquinone radicals, or

Figure 5. Fenton Reaction of Vitamin K



two-electron reduction, resulting in hydroquinones (Figures 4 and 5). Menadione was found to be more cytotoxic at higher doses than other forms of vitamin K by directly arylating nucleophiles such as glutathione and initiating one- or two-electron redox cycling. Phylloquinone and menaquinone undergo lower rates of cycling with a higher proportion of two-electron transfer.

Supporting this theory is the observation that menadione increased oxidative stress in malignant cells.^{76,77} Oxidative stress was detected by increased DNA strand breaks due to hydroxyl radicals produced by the presence of menadione in the MCF-7 cell culture.^{78,79} The hydroxyl radical arises from superoxide radical via a Fenton reaction with various transition metals (Figure 5). Also supporting this theory, antioxidants such as glutathione and enzymes (catalase and superoxide dismutase) have been shown to quench superoxide and hydrogen peroxide, decreasing the oxidative stress of menadione.⁸⁰ Reduction of oxidative stress has been shown to decrease the anticancer effect of menadione. The growth inhibition due to menadione in a Hep3B cell line was prevented by both exogenous thiols and two non-thiol antioxidants (catalase and desferoxamine mesylate).¹⁷ However, the increased effectiveness of menadione in combination with ascorbic acid illustrates the requirement of a reducing agent with the quinone for maximum effect.⁶⁶

Direct Arylation

Another aspect of an oxidative mechanism of action for vitamin K3 involves the direct arylation of thiols within the cell by menadione, resulting in the depletion of glutathione and/or sulfhydryl-containing proteins.⁶⁶ Arylation refers to the introduction of aromatic groups, such as menadione, to a molecule such as glutathione. When menadione was added to rat platelets, arylation occurred producing a menadione-glutathione conjugate.⁸¹ The ability of menadione to deplete glutathione pools in isolated rat hepatocytes was observed when K3 levels above 35 μM were reached.⁸² Menadione directly bonds to peptides at the cysteine sulfur, but not to alanine or serine residues. The decrease of sulfhydryl groups in treated cells suggests K3 might also decrease the activities of other critical sulfhydryl-containing enzymes such as protein tyrosine phosphatases as well as p34Cdc2 protein associated with cell growth.⁸³ In addition, glutathione (1 mM) added to L120 leukemia and CEM cell lines partially reduced the cytotoxicity of menadione.⁴³ A number of other studies have supported the idea of arylation with K3 as an anticancer mechanism.^{75,76,84,85} Vitamin K analogs, such as 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone, were studied in Hep3B cells and showed cell growth inhibition that was antagonized by thiols, but not by non-thiol antioxidants, also suggesting the mechanism of growth inhibition was sulfhydryl arylation.⁶⁶

Non-oxidative Model of Anticancer Activity

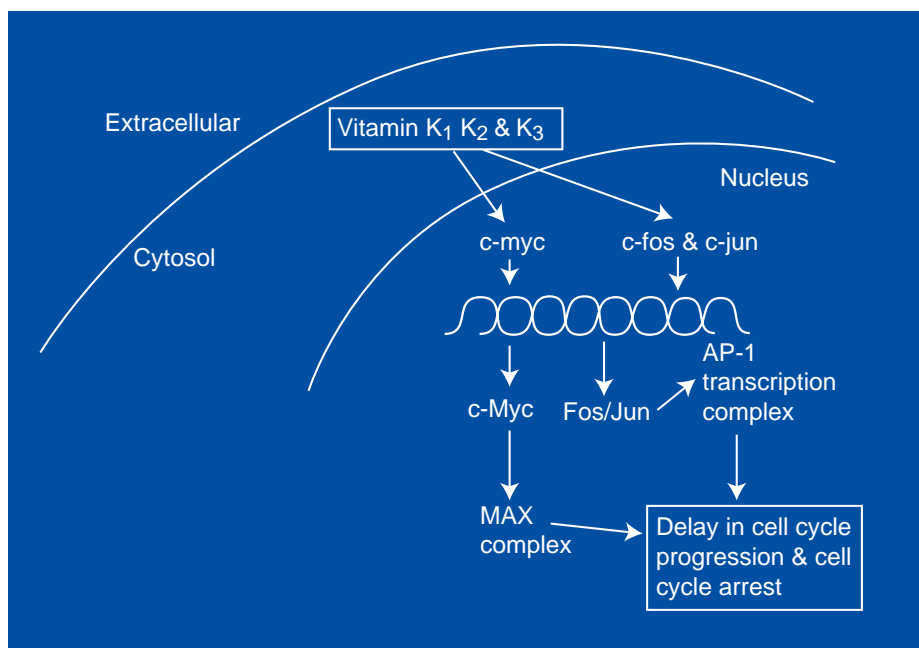
The oxidative stress produced by K3 can begin a cascade of events leading to the characteristic signs of apoptosis, cell shrinkage, DNA fragmentation, and activation of caspase-3. While there is evidence that the major anticancer mechanism of K3 at high concentrations operates by oxidative stress and arylation, both K1 and K2 must act as anticancer agents through a different mechanism. Evidence of another mechanism was demonstrated when the addition of catalase inhibited the anticancer effect of K3, but had no effect on K2.¹⁷ Catalase acts as a free radical scavenger

by neutralizing superoxide radical anion and hydrogen peroxide. Small concentrations of menadione (20 μM) led to an apoptosis frequency of 3 per high power field. Higher concentrations (60-150 μM) of K3 caused necrosis, while K2 at a concentration of 150 μM resulted in an apoptosis frequency of 1.5 with no necrosis.⁶³ K3 seems to act by two distinct mechanisms; at higher levels initiating an oxidative action and necrosis or autoschizis, while at lower levels acting by a non-oxidative mechanism inducing apoptosis.⁸⁶ K2 (and presumably K1), due to its inability to employ one-electron redox cycling, must initiate apoptosis through a non-oxidative mechanism. Another piece of evidence failing to support an oxidative mechanism for menadione is the demonstration that menadione is still cytotoxic in the presence of an iron chelator that would inhibit the production of ROS in a Fenton reaction.⁸⁷ It is the ROS products of the Fenton reaction that are thought to induce DNA breakage and cell death. The experiments above have led to the search for an alternative model to explain the non-oxidative anticancer aspects of vitamin K. The non-oxidative mechanism of K1, K2, and K3 appears to involve transcription factors.

Transcription Factors

The non-oxidative model of vitamin K action focuses on the modulation of transcription factors, which in turn induce cell cycle arrest and apoptosis. ROS, protein tyrosine kinases, and protein tyrosine phosphatases modulate transcription factors, which in turn induce the transcription of growth factors, inflammatory cytokines, and apoptotic controlling factors from proto-oncogenes. These proto-oncogene proteins,

Figure 6. Vitamin K in Cell Cycle Arrest



involved in the regulation and/or differentiation of cell growth, often have a protein kinase activity. Protein kinases phosphorylate proteins in response to intracellular and extracellular stimuli. They in turn act as the “on switch,” turning on a number of enzymes and proteins. Phosphatases, acting as the “off switch,” control the activation of protein kinases by dephosphorylating the various proteins and enzymes.

The expression of c-myc and c-fos proto-oncogenes are involved in the mechanism of vitamin K induced apoptosis, differentiation, and cell cycle arrest. The proto-oncogene myc (c-myc) codes for a nuclear protein transcription factor c-Myc (as part of a heterodimeric complex with Max protein) that activates other genes.⁸⁸ In the case of the oncogene c-myc, the complex is involved in transformation, immortalization, cell differentiation, and induction of apoptosis (Figure 6).⁸⁹

The proto-oncogene c-fos codes for a nuclear protein, which is involved in growth-related transcriptional control. The c-fos and c-jun gene products are both components of the transcription complex AP-1. AP-1 is a nuclear transcription factor that regulates growth and

tumor promotor stimuli. The c-Fos and c-Jun gene products dimerize in order to bind to the AP-1 recognition site. Jun has been associated with cellular transformation and activation of transcription.

C-myc proto-oncogene is best thought of as a modulator of cellular function, such as apoptosis, as it can promote both proliferation and apoptosis depending on its expression.^{90,91} Mutation of the proto-oncogene to the c-myc oncogene can foster malignant transformation and increase cell proliferation. The addition of vitamin K (K1, K2, and K3) to malignant cell cultures has been found to result in induction of proto-oncogenes and an increase in the level of a number of proto-oncogene proteins, c-myc, c-jun, and c-fos.^{14,92} Wu et al¹⁴ found that 50 μ M of K3 transiently induced c-fos proto-oncogene expression *in vitro* in one hour, while c-myc proto-oncogene expression was increased for 1-9 hours after treatment with concomitant increase in c-Fos and c-Myc transcription factors. These changes were associated with cell cycle delay or arrest and apoptosis. K3 is the most potent stimulator, followed by K2 then K1.¹⁷

The proto-oncogene bcl-2 has been shown to protect against apoptosis. The addition of bcl-2 to cell cultures (2B4 and FL5.12) countered the cell death imposed by the oxidative burst from K3. Bcl-2 was not able to decrease the ROS produced by K3, as measured by cyanide-resistant oxygen consumption, yet it was able to inhibit dose-related killing of the cell lines from 50 μ M-200 μ M of K3. These results suggest that ROS, acting as second messengers, signal downstream transcription factors, such as nuclear factor-kappa B (NF-kB), Fos/Jun, and others that may be protected by the antioxidant-governing activities of Bcl-2.⁹³ The transcription factor NF-kB is involved in stress-induced FasL expression. Fas is one of the important death receptors in the tumor necrosis factor superfamily. The gene encoding the ligand for Fas, designated as FasL, activates Fas by trimerization of the receptor. Activation-induced cell death is commonly mediated by the Fas/FasL system, and menadione induces Fas as well as FasL expression. Caricchio et al⁹⁴ found that a functional Fas/FasL system was needed in order to induce

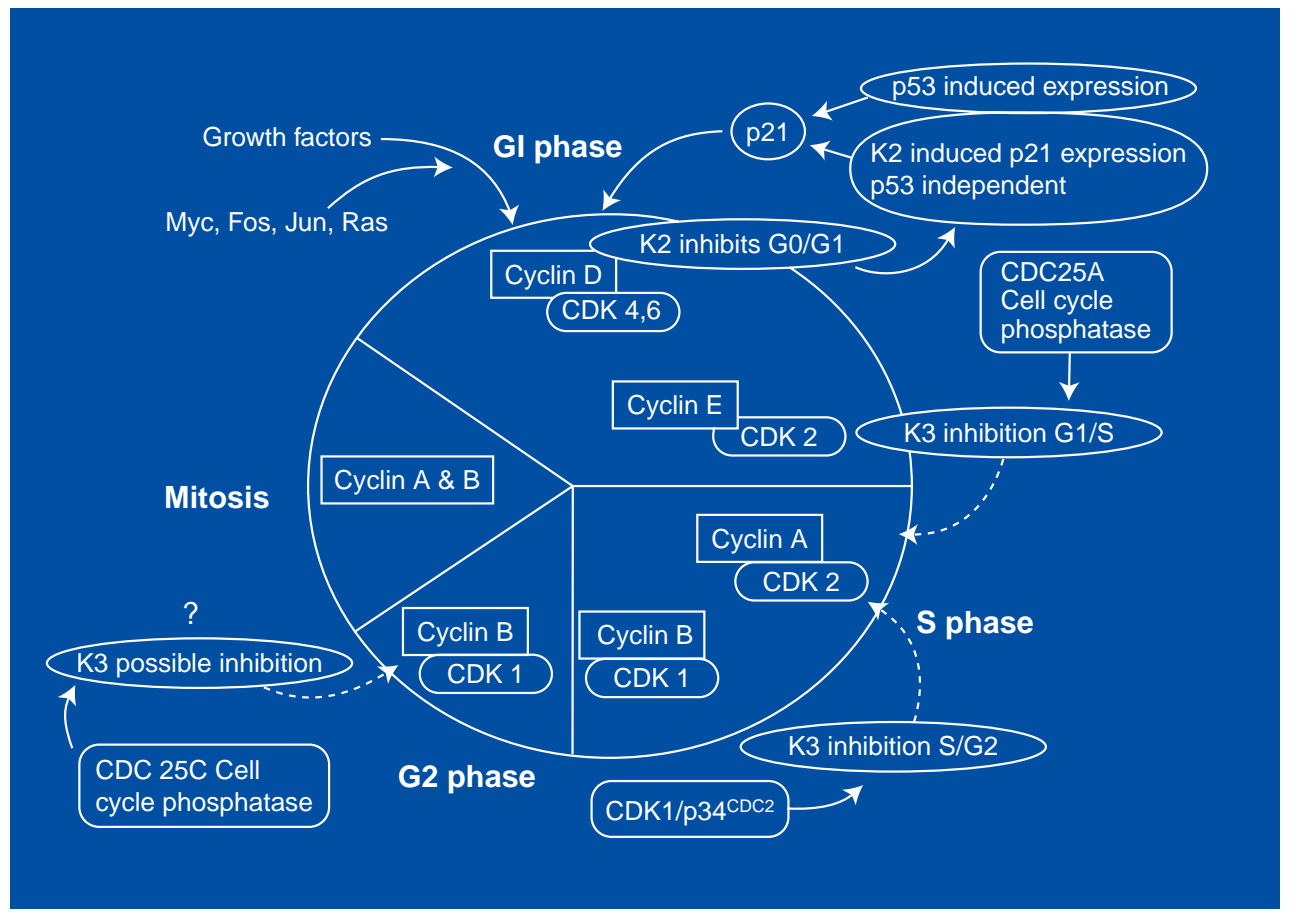
apoptosis. Experiments showed that mutant leukemia cell lines lacking a functional Fas ligand were resistant to FasL killing by menadione. It was also found that mice lacking functional FasL or expression of Fas were also resistant to the cytotoxic effects of K3.

Vitamin K3 has been shown to inhibit growth and induce apoptosis of stomach cancer cells in a dose-dependent fashion. The inhibition was found to be due to sulfhydryl arylation of critical cystine residues that mediated protein tyrosine phosphorylation. The inhibition of cell growth by K3 induced tyrosine phosphorylation of hepatocyte growth factor receptors (c-met) and epidermal growth factor receptors (EGFR), which in turn activated the RAS signaling pathway. The addition of vitamin K3 also created a sustained phosphorylation of extracellular signal-regulated kinase (ERK), part of the mitogen-activated protein kinase (MAPK) superfamily associated with cellular signal transduction, proliferation, and apoptosis. Vitamin K3 is thought to induce both protein tyrosine kinase activation from the receptor pathway and inhibit ERK protein tyrosine phosphatases (that dephosphorylate activated kinases). ERK phosphorylation has been found to be critical to not only growth factor-induced cell proliferation, but also to K3-mediated cell death. It is thought that K3 can act without ligand binding through the inhibition of protein tyrosine phosphatases.⁹⁵

Cell Cycle Arrest

The ability of vitamin K to induce cell cycle arrest and cell death may also be explained by the inhibition of protein kinases in association with a cyclin-dependent mechanism. Cyclins are regulatory proteins of the cell cycle that activate cellular maturation-promoting factors. Cyclins complex and modulate the protein kinase catalytic subunit of proteins such as p34CDC2, known alternately as cyclin dependent kinase1 (CDK1). This protein kinase is a member of the serine/threonine protein kinase family. The designation "CDC2" refers to "cell division cycle 2" at the G1 to S and G2 to M transitions. Cyclins, such as cyclin B1, have no inherent enzymatic activity;

Figure 7. Cell Cycle Regulation of Vitamin K (Modified from Hellman et al)¹⁰¹



rather, they act by means of cyclin-dependent kinases that phosphorylate serine and threonine residues on kinase cell cycle regulators. For example, cyclin B1 complexes with p34CDC2, forming the maturation-promoting factor, which in turn is essential in G1/S and G2/M transitions in the cell cycle. Phosphorylation and dephosphorylation act as on-and-off switches for the cell cycle. Dephosphorylation of p34CDC2 increases its activity.

K3 can inhibit CDKs, such as CDK1 (p34CDC2) (100 μ M for 1 hour) by hyperphosphorylating the protein.^{45,96} The addition of menadione to malignant cell culture has been shown to inhibit the cell cycle at the G1/S and S/G2 phases. Concentrations in the 25-100 μ M range have been found to delay S/G2 in a dose-dependent manner.⁴⁵ Cell division cycle 25 (CDC25) are protein-

tyrosine phosphatases critical for cell cycle progression. This family of CDC25 phosphatases is responsible for the activation of cyclin-dependent kinase CDC2 through the removal of two phosphate groups. CDC25A, required for the progression from G1 to S, has been found to be inactivated by vitamin K3, and the loss of enzymatic activity was due to modification of the active site.⁹⁷

The addition of vitamin K3 to HepG2 cells hyperphosphorylated the CDC2 kinase, inactivating the enzyme and inhibiting the cell cycle.⁹⁸ It has been proposed that menadione modifies the active sites of the CDC25 dual specificity protein phosphatases and reduces or even abolishes the dephosphorylating activity of the enzyme. Vitamin K3 binds to active sulfhydryl groups of cysteine residues at active p34CDC2

sites.⁴⁵ This action stems from binding to the catalytic domain of CDC25 phosphatase. K3 also decreased protein-tyrosine phosphatase by 2- to 3-fold⁴⁵ and suppressed the expression of proliferating cell antigen as well as cyclin B in S phase.⁹⁹

Vitamin K2 has also been shown to work at the level of the cell cycle, acting on cyclins to inhibit the cell cycle and initiate differentiation. It is a powerful inducer of differentiation in a number of myeloid leukemia cell lines in various stages of maturation. The mechanism of differentiation by K2 differs from retinoic acid. Vitamin K2 has not been found to bind retinoic acid receptors (RAR) alpha, beta, or gamma, or retinoid X receptor (RXR) alpha receptors.⁶⁸ This work with vitamin K2 implies there is an undiscovered nuclear receptor or mechanism for differentiation.

Researchers have proposed that the p21 gene may act with vitamin K2 as an additional factor in cellular differentiation. Previously it was thought that tumor suppressor genes such as p53 and BRCA1 induce the expression of the p21 gene. It was demonstrated that vitamin K2 can also stimulate p21 in a p53-independent manner.¹⁰⁰ (K2 was also shown to be unable to induce p53 in MG-63 human osteosarcoma cells, while inducing p21 gene.) MG-63 cells, shown to lack the p53 gene, were inhibited by vitamin K2 at high concentrations between 10^{-7} and 10^{-5} M/L. The elevated levels of p21 resulted in the differentiation of osteosarcoma cells.

The action of vitamin K2 in cell cycle arrest acts at the G1/S transition. When K2 transcriptionally activates the p21 protein, it complexes and inhibits the phosphorylation of G1 cyclin-dependent kinases in the cell cycle. This results in the arrest of cells in the G0/G1 phase of the cell cycle.

Conclusion

Vitamin K, in all its various forms, has been shown to have anticancer effects. Vitamin K cancer research has focused on two basic mechanisms to explain these effects. The older mechanism relies on an oxidative effect produced by the one-electron cycling of vitamin K3 that surpasses the oxidative capacity of the cancer cell, leading

to death. Other mechanisms have been proposed due to the anticancer effect of vitamin K forms that either do not readily cycle (K1 and K2) or that are at levels that do not initiate cycling. These clues to another mechanism have led researchers to discover an alternative mechanism of action that acts at the level of protein kinases and phosphatases. Vitamin K has been found to act on proteins such as myc and fos, which in turn leads to growth arrest and death. Cell cycle arrest has also been found to be initiated by phosphatases at the level of cyclins, which are critical in the cell cycle.

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