Vitamin K enhancement of sorafenib-mediated HCC cell growth inhibition *in vitro* and *in vivo*

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The multikinase inhibitor sorafenib is the first oral agent to show activity against human hepatocellular carcinoma (HCC). Apoptosis has been shown to be induced in HCC by several agents, including sorafenib as well as by the naturally occurring K vitamins (VKs). As few nontoxic agents have activity against HCC growth, we evaluated the activity of sorafenib and VKs, both independently and together on the growth of HCC cells *in vitro* and *in vivo*. We found that when VK was combined with sorafenib, the concentration of sorafenib required for growth inhibition was substantially reduced. Conversely, VK enhanced sorafenib effects in several HCC cell lines on growth inhibition. Growth could be inhibited at doses of VK plus sorafenib that were ineffective with either agent alone, using vitamins K1, K2 and K5. Combination of VK1 plus sorafenib induced apoptosis on FACS, TUNEL staining and caspase activation. Phospho-extracellular signal-regulated kinase (ERK) levels were decreased as was myeloid cell leukemia sequence 1 (Mcl-1), an ERK target. Sorafenib alone inhibited growth of transplantable HCC *in vivo*. At subeffective sorafenib doses *in vivo*, addition of VK1 caused major tumor regression, with decreased phospho-ERK and Mcl-1 staining. Thus, combination of VK1 plus sorafenib strongly induced growth inhibition and apoptosis in rodent and human HCC and inhibited the RAF/mitogen-activated protein kinase kinase/ERK pathway. VK1 alone activated PKA, a mediator of inhibitory Raf phosphorylation. Thus, each agent can antagonize Raf: sorafenib as a direct inhibitor and VK1 through inhibitory Raf phosphorylation. As both agents are available for human use, the combination has potential for improving sorafenib effects in HCC.

Human hepatocellular carcinoma (HCC) is the fifth commonest cause of cancer deaths worldwide¹ and is increasingly frequent in the United States,² with ~15,000 new cases annually.³ It arises on the basis of cirrhosis in most patients, so that patients have two diseases of their liver, namely cirrhosis and cancer. The presence of a chronically damaged or cirrhotic underlying liver renders chemotherapy unsafe for many patients, because their fragile livers cannot always tolerate toxic chemotherapy. Thus, there is the need for less toxic and effective therapy for HCC. The newer cell cycle inhibitors and antiangiogenic agents that are currently under evaluation in many clinical trials have the potential for filling this need.

Sorafenib is a multikinase inhibitor that was originally developed as an inhibitor of Raf-1, but it was subsequently shown to inhibit multiple other kinases, including platelet-derived growth factor, vascular endothelial growth factor receptors 1 and 2, c-Kit and FLT3.⁴ It has broad activity against various tumor cell lines *in vitro* and in xenograft models.⁵ Antitumor effects of sorafenib in renal cell carcinoma and in hepatoma have been ascribed in part to antianangiogenic actions of this agent through inhibition of multiple growth factor receptors⁶ as well as by inhibition of tumor cell growth signaling pathways. Preliminary evidence of single-agent activity has also been observed in malignant melanoma,⁷ pancreas cancer⁸ and hematological malignancies⁹ amongst others. As the clinical application of sorafenib evolves, there is increasing interest in defining the several mechanisms underlying its antiproliferative activity as well as in examining the effects of the agent in combination with other drugs. Furthermore, sorafenib causes multiple human toxicities, including use-limiting anorexia, GI bleeds and hand-foot syndrome.¹⁰,¹¹ Modulation of its actions that result in lessening of the toxicities is a desirable goal.

VK Vitamins (VKs) are fat-soluble vitamins that are involved in blood coagulation and bone metabolism.¹²,¹³ In recent years, their antitumor effects have also been examined.¹⁴,¹⁵ They have been shown to suppress cancer growth and induce apoptosis and differentiation in various cancer cells, including leukemia,¹⁶ melanoma¹⁷ and HCC cells *in vitro* and *in vivo*.¹⁸,¹⁹ There are several forms of VK: VK1 (phytonadione), which is produced by plants and is used to treat human coagulation disorders; VK2 (menaquinone), which is produced by certain bacteria and also occurs
naturally and in the human gut and is used to treat osteoporosis and HCC, synthetic VK3 (menadione), which is a short-chain chemically synthesized form that induces redox cycling and is toxic in humans and VK5, another short-chain synthetic analog with inhibitory actions on both cells and bacteria. The natural VK1 and VK2 are thought to be without toxicity in adult humans. It has been shown that natural VKs can inhibit HCC cell growth and induce apoptosis in vitro and in vivo26 and possibly also in humans with HCC.22,23 VK2 has also been shown to cause PKA activation,26 which in turn is a known mediator of inhibitory Raf phosphorylation.27

We reasoned that as both sorafenib and VKs are available for human use and because they each independently have been shown to both inhibit Raf and growth and induce apoptosis in HCC cells, then the combination might be expected to be more potent than either agent alone. We show that combining sorafenib with natural VKs more effectively inhibits HCC cell line growth than either agent alone. By therapeutically targeting the MAPK pathway, these combined agents dramatically induce apoptosis in cells in vitro and inhibit HCC tumor growth in vivo. Furthermore, we show that combined sorafenib plus vitamin K induced HCC cell death via inhibition of phospho-extracellular signal-regulated kinase (ERK) and activation of the extrinsic apoptotic pathway. These results lay the groundwork for possible future clinical evaluation of this combination.

**Material and Methods**

**Cell lines**

Human HCC cell lines (Hep 3B, PLC/PRF/5 and Hep 40) were obtained from American Type Culture Collection (Rockville, MD), and JM1 transplantable rat HCC cell line was originally a gift of G. Michalopoulos, University of Pittsburgh and used by us previously.28 They were cultured in Minimum Essential Medium Eagle (MEM) containing 10% fetal bovine serum in 5% CO2 at 37°C. Unless otherwise indicated, cell culture reagents were obtained from Life Technologies (Gaithersburg, MD).

**Compounds**

Sorafenib [N-(3-trifluoromethyl-4-chlorophenyl)-N'-(4-(2-methylcarbamoyl pyridin-4-yl) urea] was synthesized at Bayer Corporation (West Haven, CT). Sorafenib was dissolved in 100% DMSO (Sigma, St. Louis, MO) and diluted with MEM to the desired concentration with a final DMSO concentration of 0.1% for in vitro studies. Vitamin K1 was purchased from Sigma-Aldrich Chemical, MO and dissolved in 99.9% ethanol at a stock concentration of 50 mM and then diluted to appropriate concentrations with medium in using. Compounds were diluted with MEM to the desired concentration with final DMSO or ethanol concentration of 0.1% for in vitro studies. DMSO and ethanol were added to cultures at 0.1% (V/V) as a solvent control.

**Cell viability**

Cells were plated at a concentration of ~2 × 10^4 cells per well in 24-well plates. Twenty-four hours after plating, the medium was replaced with fresh MEM containing vitamin K, sorafenib or a combination of the two agents at the indicated concentrations. Three days after the treatment, the medium was removed, and the plates were stored at ~80°C until the day of the assay. The cell number was estimated by a DNA fluorometric assay using the fluorochrome Hoechst 33258.

**In situ end labeling of fragmented DNA (TUNEL)**

Cells were cultured on chamber slides and treated with compounds as described above. After 48 hr of treatment, cells were fixed with 10% buffered formalin at room temperature, washed with phosphate-buffered saline (PBS) and air-dried. Fragmented DNA was detected by an in situ end labeling kit (ApopTag kit, Oncor, Gaithersburg, MD) according to the manufacturer's protocol. Briefly, digoxigenin-labeled dUTP was incorporated at the 3'-OH ends of the fragmented DNA by terminal deoxynucleotidyl transferase, the anti-digoxigenin antibody conjugated with peroxidase was then applied and the peroxidase activity was revealed by 3-amino-9-ethylcarbazol. Nuclei were then counterstained lightly with hematoxylin.

**Immunoblot analysis**

Cells were plated in 100-mm tissue culture dishes with a density of 5 × 10^5/dish overnight. The cells were treated in the next morning and harvested in different time. After harvest, the cells were washed with cold PBS and then lysed in 100 μl lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% Triton X-100, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin and 10 mg/ml aprotinin). Whole cell extracts (20 μg) were resolved on a 10% SDS-PAGE and transferred onto Hybond-PVDF membranes (Amersham, Arlington Heights, IL). Membranes were blocked using Tris-buffered saline with Tween-20 (150 mM NaCl, 10 mM Tris-HCl, pH 8.0 and 0.05% Tween-20) containing 1% BSA for 1 hr and then probed with primary antibody for 2 hr. After washing four times with Tris-buffered saline with Tween-20 (TBST buffer), the membranes were probed with horseradish peroxidase-conjugated secondary antibody to allow detection of the appropriate bands using enhanced chemiluminescence (Amersham). The antibodies used in these experiments (PARP, Caspase-3, 8, 9, ERK1/2, myeloid cell leukemia sequence 1 (Mcl-1) and Actin) were obtained from Santa Cruz Biotechnology, Santa Cruz, CA or Cell Signaling, Waltham, MA.

**Apoptosis determination**

Annexin V-FITC kit (BD Biosciences, San Diego) was used to measure the percentage of apoptosis induced by vitamin K1 or sorafenib. After treatment for 36 hr, cells were harvested, washed with PBS at 4°C and then resuspended in
100 µL binding buffer (1 × 10^6 cells/ml) containing 5 µl of Annexin V-FITC and 10 µl of PI. After incubation away from light for 10–15 min at room temperature, stained cells were then analyzed by flow cytometry.

**Drug synergy evaluation**

The potential synergistic, additive or antagonistic effects of VK plus sorafenib used in combination were assessed experimentally and computationally using methods as described by Chou and Chou and Talalay, implemented in Calcsyn software (Biosoft, UK). The Chou and Talalay approach takes into account drug potency as well as the relationship between dose and response for each drug. Results are reported as the combination index (CI). Values of CI < 1, CI = 1 and CI > 1 imply synergy, additivity and antagonism, respectively. An isobologram based on an extension of the Lowe additivity model is also provided. Similar to the CI values, values on the isobologram below the line of additivity represent combinations where synergy is present, values close to the line would represent additivity and values above the line represent antagonistic effects.

**In vivo transplantable rat hepatoma growth and treatments**

A total of 1 × 10^6 JM-1 cells per rat were injected under direct vision into mesenteric vein feeding into the portal vein. Sorafenib and/or vitamin K1 injections were started 3 days after the implantation of JM1 cells, as performed previously. The in vivo JM1 transplantable HCC experiment was divided into four groups with five rats in each group: controls (DMSO and/or ethanol, 100 µl each), sorafenib (1.25 mg/kg body weight), vitamin VK1 (2 mg/kg body weight) and combined sorafenib plus vitamin VK1 treatment. The compounds were injected intraperitoneally daily for 8 consecutive days. Three days after the last injection, the animals were sacrificed and the tumors were counted, weighed and the JM1 rat HCC cell line (Fig. 1b). We also tested whether other VKs could enhance sorafenib-mediated HCC growth inhibition and found that vitamins K2 and K5 were similar in this respect to vitamin K1 (Fig. 1c).

We then examined whether the effects of simultaneous addition of vitamin K1 to sorafenib were additive or more than additive. Figure 1d represents the isobologram for the combination of varying doses of sorafenib and fixed dose of vitamin K1. Isobologram calculations indicated that there was observed synergy in the combination of sorafenib and vitamin K1, because all values in Figure 1d are well below the line of additivity. Additionally, CIs were computed for each combination, and values were ranged from 0.39 to 0.77. Synergy is indicated for CI < 1, additivity for CI = 1 and antagonism for CI > 1. Given that CI is consistently <1 for all sorafenib concentrations plus vitamin K1, the corresponding concentration reduction indices were computed, yielding 2.3- to 6.7-fold concentration reduction potential for sorafenib.

**Induction of apoptosis by sorafenib plus vitamin K1**

As combination of sorafenib plus vitamin K1 caused a significant reduction in cell proliferation, the underlying mechanisms were investigated. First, TUNEL staining of treated cells showed the presence of apoptosis after this combination treatment, compared to either agent alone (Fig. 2a). Pretreatment with ZVAD, a pan-caspase inhibitor, significantly blocked the induced apoptosis, as measured by TUNEL staining. To confirm the induction of apoptosis by this combination, cells were treated with the agents individually or in combination and examined by Annexin V/propidium iodide staining and subsequent FACS analysis (Fig. 2b). At the concentrations tested, neither sorafenib nor vitamin K1 elicited significant apoptosis as a single agent, but the combination induced apoptosis in 43% of the cells. Pan-inhibition of caspase activity using ZVAD significantly reduced the cell death percentage (Figs. 2b and 2c). These results show that combination of sorafenib plus vitamin K1 caused apoptosis, which was inhibited by a caspase antagonist.

**Involvement of the extrinsic pathway in sorafenib plus vitamin K1-mediated apoptosis**

To further examine the processes of cell death induced by this combination, we analyzed cell extracts for expression of biological markers of apoptosis. The combination drug treatment resulted in marked cleavage of pro-caspase-3 and poly(ADP-ribose) polymerase (PARP) induction, whereas low concentrations of the individual agents did not (Fig. 3a). The upstream caspases of caspase-3 were next examined.
Treatment with combination of sorafenib plus vitamin K1 resulted in caspase-8 cleavage, but no pro-caspase-9 activity could be detected in the same treated samples (Fig. 3b). These findings suggest that caspase-8 signaling and the extrinsic pathway were involved in the combination-induced apoptosis. No changes were seen in the levels of antiapoptotic Bcl-2 or of Bcl-xL, but the combination treatment caused a decrease in the levels of Mcl-1, but not of survivin (Fig. 3c).

**Inhibition of RAF/MEK/ERK signaling by sorafenib, vitamin K1 and the combination**

Raf kinases are key regulators of the mitogen-activated protein kinase kinase (MEK)/ERK cascade, and upregulated signaling through this pathway has an important role in cancer cell growth. As sorafenib was synthesized as a RAF inhibitor, we examined the phosphorylation levels of key Raf substrates in the pathway by Western blot in cells treated with various doses of sorafenib, vitamin K1 or by the combination. Neither sorafenib nor vitamin K1 alone could inhibit ERK phosphorylation at the low concentrations used (Fig. 4a), whereas the combination of these two agents at these lower concentrations caused a reduction in phosphorylated ERK levels (Fig. 4a), but total ERK levels were unchanged. As antiapoptotic Mcl-1 levels have been shown to be controlled by ERK activity, we also examined whether a decrease in phospho-ERK levels was associated with a change in Mcl-1 levels. We found that the combination treatment caused a decrease in both phospho-ERK and Mcl-1 (Fig. 4b). We then examined various concentrations of sorafenib alone and found that higher doses caused a decrease in Mcl-1 levels (Fig. 5a) as well as the expected decreases in phospho-ERK levels, as shown previously. As vitamin K2 has been shown to activate PKA in hepatoma cells and PKA can mediate phosphorylation on Raf at sites that inhibit its actions, we...
examined their levels in cells that had been treated with vitamin K1 alone and found increased levels of both phospho-Raf as well as increased levels of the Raf inhibitory phospho-Raf (ser43) and phospho-Raf (ser259). Phospho-ERK levels were also decreased, but not as much as with sorafenib alone (Fig. 5b). Furthermore, we also found that vitamin K1-
induced PKA and Raf phosphorylation could be blocked by PKA inhibitor (Fig. 5c).

**Sorafenib plus vitamin K1 inhibit HCC growth in vivo**

JM1 rat HCC cells were grown in vitro and then seeded into the liver via the portal vein (Material and Methods). After the injected cells were allowed to establish and grow, rats were treated with either vitamin K1 or sorafenib or the combination of both agents. Ten days after the first injection of compounds, the rats were sacrificed, and the tumors were excised and weighed. The total weight of the tumors from each rat liver was measured, and the ratio of tumor weights to liver weights was quantitated, as shown in Figure 6c. Vitamin K1 inhibited tumor growth minimally, whereas sorafenib alone could inhibit in vivo tumor growth as noted previously, depending on the dosage used. However, the combination of vitamin K1 together with sorafenib at a low dosage that was minimally effective when used as a single agent caused major tumor shrinkage when given as a combination (Fig. 6a). As we had shown above that the combination caused a decrease in phospho-ERK and Mcl-1 levels in cells in culture (Figs. 4a and 4b), we stained the tumors that could be seen at lower sorafenib plus vitamin K1 combination doses and we found decreased staining for p-ERK and Mcl-1 and increased phospho-PKA in the tumors of treated rats, compared to the surrounding liver in the same animals (Fig. 6b), consistent with the idea that similar mechanisms were involved in vivo as were found in vitro.

**Discussion**

HCC typically arises on the basis of cirrhosis and responds poorly to conventional cytotoxic chemotherapy. The latter is often poorly tolerated by a liver that has been damaged by chronic viral or ethanol exposure. This has led to a search for novel approaches to therapy, including the targeting of the EGF receptor (erlotinib), VEGF (bevacizumab) or the VEGF receptor (sorafenib, sunitinib) and the convergent RAF/MEK/ERK pathways by new therapeutic agents. The RAF/MEK/ERK cascade is one of the principal RAS-regulated pathways. Raf expression has been reported to be increased in human HCC, and sorafenib was synthesized to molecularly target RAF in this vital pathway and has been shown to have antitumor activity against renal cell cancer and HCC. Sorafenib seems less effective for treating other types of cancer, although preliminary data have shown some activity for sorafenib in pancreas cancer and some other
tumor types. However, it also has many toxicities and a variable but large percent of patients need to be dose-reduced or stop taking the drug for this reason. This was the stimulus for our search for agents that could be combined with sorafenib to enhance its HCC growth-inhibitory actions, given that it can enhance overall survival in HCC patients, albeit by only a few weeks. Given that vitamin K1 appears to be without toxicity in adult humans and that vitamin K can enhance HCC cell PKA phosphorylation, and phospho-PKA in turn has been shown to phosphorylate and modulate Raf activity in HCC cells, we examined whether these two agents might have additive or superadditive effects on HCC cell growth.

We previously found preliminary evidence that addition of vitamin K1 could enhance sorafenib-mediated apoptosis in HCC cells in vitro. The present studies examined this combination in greater detail and begin to elucidate the mechanism(s) responsible for this superadditive phenomenon. We show here for the first time that combination of vitamin K1 with low and clinically relevant concentrations of sorafenib inhibited growth and induced apoptosis in several human HCC cell lines, in vivo and in a rodent HCC cell line, both in vitro and in vivo. This finding has clinical possibilities, as it suggests that the combination might be a candidate for clinical application, either to permit use of lower and less toxic sorafenib doses or to add to standard sorafenib doses to enhance clinical responses, that have so far been meager.

Prior studies have shown that sorafenib in the 10 μmol/l range, which is at pharmacologically achievable concentrations, induced cell death in human leukemic cells, HCC cells and pancreatic cancer cells. The results of our study show that low concentrations of sorafenib (2.5 μM) or vitamin K1 (50 μM) when used as a single agent did not cause growth inhibition or apoptosis. However, treatment of HCC cells with low

Figure 6. Inhibition of HCC tumor growth in vivo by sorafenib plus vitamin K1. (a) After injection of JM1 hepatoma cells into the liver via a portal vein tributary, rats were treated with either solvent control, vitamin K1, sorafenib or combination of vitamin K1 plus sorafenib. They were then sacrificed, and whole livers were isolated as described in Material and Methods. A tiny tumor in combination group is labeled with arrow. (b) Representative histology and histochemistry slides from the tumors and livers stained by H and E and for phospho-ERK, Mcl-1 and Phospho-PKA. Original magnification ×10. (c) The in vivo JM-1 cell transplantable hepatoma experiment was divided into four groups: (i) vehicles, (ii) sorafenib, (iii) vitamin K1 and (iv) combined sorafenib/vitamin K1 treatment. The compounds were injected intraperitoneally daily for 8 consecutive days. Three days after the last injection, the animals were sacrificed, and the tumors were excised. The ratio of total weight of the tumors and liver from each rat liver was counted. The results are expressed as the mean ± standard deviation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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concentrations of both sorafenib (2.5 μM) plus vitamin K1 (50 μM) resulted in cell growth inhibition and caused apoptosis (Figs. 1–3) as well as significantly inhibiting the phosphorylation of ERK (Fig. 4a). Therefore, vitamin K1 seems to add to sorafenib inhibition of the MEK/ERK pathway. Apoptosis induced by sorafenib plus vitamin K1 was caspase dependent, because pretreatment with pan-caspase inhibitor could dramatically block the apoptosis induced by sorafenib plus vitamin K1. The apoptotic signaling pathways are generally divided into two types: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway.48 The extrinsic pathway involves cell surface death receptors, such as tumor necrosis factor or Fas, which upon binding of their ligands, initiate signaling to activate caspase-8, which cleaves caspase-3 directly to induce apoptosis. The intrinsic pathway involves mitochondrial changes and triggers the release of cytochrome c, which in turn activates caspase-9 and then caspase-3.49 Our findings suggest that a central mechanism of sorafenib plus vitamin K1-mediated apoptosis in HCC cells involves activation of the extrinsic apoptosis pathway.

It was recently reported that activated ERK plays an important role in controlling levels of the antiapoptotic protein Mcl-1.74 Here, we demonstrated that actions of sorafenib or vitamin K1 (at very high concentrations) alone can result in decreased phospho-ERK levels, but vitamin K1 at low concentrations significantly added to low and minimally effective concentrations of sorafenib to result in inhibition of the MEK/ERK pathway, a decrease in Mcl-1 levels and induction of apoptosis. Sorafenib alone at higher concentrations could cause a decrease in Mcl-1 levels, but much lower concentrations of sorafenib also caused this, when vitamin K1 was present (Figs. 4b and 5a). Tumors were grown in the animal livers after injection into the mesenteric vein under direct visualization. Vitamin K1 alone had minimal effects at the doses used. As reported for HCC in other models,49 sorafenib could inhibit tumor growth. However, when subeffective doses of sorafenib were used, addition of vitamin K was able to cause complete disappearance of tumors, as judged both by direct inspection of the excised livers (Fig. 6a) and on microscopic examination (Fig. 6b). Furthermore, we found that the tumors from treated animals had decreased staining for both phospho-ERK and Mcl-1, consistent with the Western blot results from treated cells in culture.

The mechanisms for the growth-inhibitory effects of vitamin K1 are not yet clear. We considered this combination after reports that VK actions can result in phosphorylation and enhancement of PKA activity.21,41 As PKA has been shown to mediate inhibitory phosphorylation of Raf, the key target of sorafenib, it seemed possible that the combination might cause increased growth inhibition by each agent inhibiting Raf through different mechanisms: sorafenib as a direct Raf inhibitor and vitamin K1 by inducing inhibitory Raf phosphorylation. Preliminary evidence is presented for the latter.

In conclusion, our data indicate that: (i) combination of sorafenib plus vitamin K1 can decrease the concentrations of sorafenib that were needed to inhibit HCC cell growth and induce apoptosis; (ii) vitamin K1 enhanced sorafenib-mediated inhibition of the MEK/ERK pathway and activation of caspase pathway; (iii) the possible mechanisms involved in sorafenib plus vitamin K1-induced apoptosis of HCC cells involve the caspase-dependent extrinsic apoptotic pathway, likely via inhibition of Mcl-1 and (iv) sorafenib-mediated tumor cell growth inhibition in vivo was enhanced by the nontoxic vitamin K1.

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