An Attempt to Evaluate the Effect of Vitamin K₃ Using as an Enhancer of Anticancer Agents

Sumio MATZNO,*^{a,b} Yuka YAMAGUCHI,^a Takeshi AKIYOSHI,^a Toshikatsu NAKABAYASHI,^a and Kenji MATSUYAMA^c

^a School of Pharmacy and Pharmaceutical Sciences, Mukogawa Women's University; ^b The Joint Center of Industry and Mukogawa Women's University for Developing Receptor-targeting Anticancer Agents; 11–68 Kyuban-cho, Koshien, Nishinomiya, Hyogo 663–8179, Japan; and ^c Department of Clinical Pharmacy, Kyoritsu University of Pharmacy; 1–5–30 Shibakoen, Minato-ku, Tokyo 105–8512, Japan.

Received December 1, 2007; accepted March 4, 2008; published online March 6, 2008

The possibility of vitamin K₃ (VK₃) as an anticancer agent was assessed. VK₃ dose-dependently diminished the cell viability (measured as esterase activity) with IC₅₀ of 13.7 μ M and Hill coefficient of 3.1 in Hep G2 cells. It also decreased the population of S phase and arrested cell cycle in the G₂/M phase in a dose-dependent manner. G₂/M arrest was regulated by the increment of cyclin A/cdk1 and cyclin A/cdk2 complex, and contrasting cyclin B/cdk1 complex decrease. Finally, combined application demonstrated that VK₃ significantly enhanced the cytotoxicity of etoposide, a G₂ phase-dependent anticancer agent, whereas it reduced the cytotoxic activity of irinotecan, a S phase-dependent agent. These findings suggest that VK₃ induces G₂/M arrest by inhibition of cyclin B/cdk1 complex formation, and is thus useful as an enhancer of G₂ phase-dependent drugs in hepatic cancer chemotherapy.

Key words vitamin K₃; hepatic cancer; G₂/M arrest; etoposide

Vitamin K (Fig. 1), an essential nutrient, has an important role in normal blood coagulation systems from post-translational modification.¹⁾ It acts as a cofactor of a number of plasma proteins such as prothrombin, factor VII, IX and X.

In recent years, several investigations have shown that vitamin K also possesses anticancer activity.^{2,3)} The action mechanism was mentioned in two different fields. Hemostatic proteins, such as thrombin, fibrin and tissue factors, in fact, play dual roles in both thrombosis formation and cancer progression.^{4,5)} On the other hand, Lamson and Plaza reviewed that vitamin K₃ (VK₃) directly inhibited cancer cell growth *in vivo* and *in vitro*,³⁾ causing growth inhibition of Hep G2⁶⁾ and Hep 3B⁷⁾ human hepatoblastoma.

Several action theories have been proposed in the growth inhibitory and cytotoxic effects of VK₃. Historically, the main effect is believed to be due to oxidative stress *via* redox cycling of the quinone to produce reactive oxygene species,^{8,9)} while a lower concentration of VK₃ induces apoptosis by a non-oxidative mechanism.¹⁰⁾ In hepatocytes, VK₃ increases the level of p21^{Cip1}, which induces the cell cycle to G₀/G₁ arrest.¹¹⁾ In addition, previous reports showed that several synthesized vitamin K analogs regulated the G₁/S phase by antagonizing Cdc25A directly.^{12–14)}

In therapeutic use, a lower dosage is required for practical applicability. For this reason, we evaluated the possibility of VK₃ in non-oxidative conditions in hepatic cancer cells. Our

observations indicated that VK₃-induced apoptosis intensity is insufficient for anticancer monotherapy; however, it has potential as a chemotherapeutic enhancer in combination with G₂/M-selective topoisomerase II inhibitor.

MATERIALS AND METHODS

Cells and Reagents Hep G2 human hepatoblastoma cells were maintained with Williams' E (WE) medium supplemented with 10% fetal bovine serum (FBS), 50 IU/ml penicillin G and 50 μ g/ml streptomycin. Protein G-conjugated Sepharose 4B, irinotecan and etoposide were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, U.S.A.). Peroxidase-conjugated anti-mouse IgG was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL, U.S.A.). All other materials were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Evaluation of Growth Inhibitory Potential Cell Counting Kit-F (Dojindo Laboratories, Kumamoto, Japan) was used to assess cell viability.¹⁵⁾ Briefly, cells were seeded onto collagen-coated 96 well culture plates at a density of 1×10^4 cells/well and incubated for 24 h. The cells were treated with VK₃ for a further 24 h. They were then washed with PBS and allowed to react with calcein-AM for 30 min. The released fluorescent calcein was measured in a CytoFluor[®] Plate Reader (PerSeptive Biosystems, Foster City, CA, U.S.A.) using an excitation wavelength of 490 nm/emission 515 nm.

Flow Cytometry HepG2 was seeded onto collagen-coated 100 mm cell culture dishes at a density of 1.5×10^6 cells/dish and cultured for 24 h. They were then incubated with serum-starved WE for a further 24 h to synchronize the cell cycle to G₁ phase. The medium was then changed to FBS-containing WE and the cells were treated with VK₃ (3 to 30 μ M) for 8 h, and subsequently incubated with 10 μ M BrdU for 30 min. Trypsinized cells were collected by centrifugation (750 *g*, 10 min at 4 °C) and fixed in ice-cold 70% ethanol overnight. They were resuspended in 0.1% Na₂B₄O₇

Fig. 1. Chemical Formulae of Vitamin Ks

* To whom correspondence should be addressed. e-mail: smatzno@mukogawa-u.ac.jp

solution and allowed to stand for 2 min. After neutralization with hydrochloric acid, they were centrifuged and the pellet was reacted with FITC-conjugated anti-BrdU antibody (1/200 dilution with 0.5% Tween 20 in PBS) for 30 min, followed by propidium iodide (0.5 mg/ml) for 10 min under dark conditions. Flow cytometric profiles were determined with FACSCalibur analyzer and CELLQuest software (Becton Dickinson Immunocytometry Systems, Mountain View, CA, U.S.A.) at an excitation of 488 nm argon laser.

Detection of Cyclins and Cell Cycle-Dependent Kinases G₁-arrested Hep G2 were incubated with VK₃ for 8 h as described above. They were harvested with lysis buffer (0.5% SDS, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 10 μ M Na₂MnO₄, 1 mM PMSF, 10 mM leupepin and 20 mM Tris; pH was adjusted at 7.5 with HCl). The lysate was then immunoprecipitated using anti-phosphotyrosine or anti-cyclins with protein G Sepharose 4B for 17 h at 4 °C by conventional methods. Electrophoresis was performed on 12.5% T gel and the separated proteins were electrotransferred to the membrane at 150 mA for 1 h. After washing twice with TBST (0.1% Tween 20, 150 mM NaCl and 10 mM Tris-HCl, pH 7.4), the blots were blocked in 5% skim milk for 1 h. Next, each primary antibody against cyclin-dependent kinase (cdk, 1 : 500 dilution) was applied for 17 h at 4 °C. After washing, peroxidase-conjugated anti-mouse IgG (1 : 5000 dilution) was treated for more 1 h, followed by the reaction of enhanced chemiluminescence (Amersham Pharmacia Biotech) to detect antigens.

Enhancement of Antitumor Agents The cells were plated onto a collagen-coated 96 well plates and cultured for 24 h. After washing with PBS, they were treated with irinotecan or etoposide for a further 24 h in the presence of 5 μ M VK₃. Cell viability was assessed using Cell Counting Kit-F (Dojindo Laboratories, Kumamoto, Japan) as described above. The released fluorescent calcein was measured as a intracellular esterase.

Statistical Analysis In order to evaluate the cell population of each cell-cycle phase, Williams' multiple comparison followed by discriminant analysis was applied. Regarding the assessment of synergistic action between anticancer drugs and VK₃, data were analyzed through two-way analysis of variance (ANOVA). Results are expressed as mean $\pm$ S.D. and a *p* value of less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

The authors first assessed the effect of VK₃ on the cell viability of Hep G2 using cytosolic esterase activity (Fig. 2A). VK₃ dose-dependently diminished the cell viability with IC₅₀ value of 13.7 μ M. The Hill coefficient value was 3.1, and the concentration-dependent slope was steep. We next evaluated the effect of VK₃ on the cell cycle of Hep G2 human hepatoblastoma. As shown in Fig. 2B, VK₃ decreased the population of the S phase and arrested the cell cycle at the G₂/M phase in a dose-dependent manner. G₂/M arrest was displayed more clearly using discriminant analysis (Fig. 2C).

Regulation of Cell Cycle by VK₃ To clarify the action mechanism of G₂/M arrest, we next examined the activation of cyclin-dependent kinases (cdk). Multiple phosphorylated sites play an important role in both cdk activation and inacti-

Fig. 2. Growth Inhibition of Hep G2 Cells by VK₃

(A) Concentration-dependent cytotoxicity of VK₃ in Hep G2 cells. Cells were treated with VK₃ for 24 h and intracellular esterase activity was assessed as an index of cell viability. (B) Effect of VK₃ on cell cycle progression. G₁-arrested Hep G2 were incubated with 3–50 μ M of VK₃ for 8 h. Double-stained cells with BrdU and PI were analyzed with flow cytometry. Values represent the mean $\pm$ S.D. (*n* = 7). Significantly different from control; * *p* < 0.05, ** *p* < 0.01. (C) Discriminant analysis of cell cycle arrest of Hep G2 to G₂/M-phase.

vation. Threonine phosphorylation in the T-loop, mediated by cdk-activating kinase, is essential for activation of the cyclin/cdk complex.^{16,17)} On the other hand, the cyclin/cdk2 complex in the G₂ phase is downregulated by phosphorylation at Tyr15, which is situated in the ATP-binding site of cdk2.¹⁸⁾ Since VK₃ acts in G₂/M arrest, we investigated the effect of VK₃ on tyrosine phosphorylation in cdk.

Eight hours after VK₃ addition, the cells were harvested and Tyr-phosphorylated cdk were detected by immunoblot analysis. Tyr-phosphorylated cdk1, 2 and 4 were detected in hepatocytes treated with VK₃ at 10 μ M; however, phosphorylation was depressed at higher concentration in cdk2 and 4 (Fig. 3A). Concomitantly with Tyr phosphorylation, cyclin B was gradually decreased and cyclin D was completely diminished by VK₃ treatment (Fig. 3B). We further demonstrated the formation of cyclin/cdk complexes (Fig. 3C). In VK₃-treated hepatocytes, cyclin A/cdk1 and cyclin A/cdk2 complex dose-dependently increased, whereas the cyclin D/cdk4 and cyclin B/cdk1 complex was decreased. These findings indicate that VK₃ accelerates S phase turnover by the activation of cyclin A/cdk2 and induces G₂/M arrest by the inhibition of cyclin B/cdk1 complex formation.

Enhancement of VK₃ on G₂/M Phase-Dependent Chemotherapy Our findings suggest that VK₃ would be

Fig. 3. Cell Cycle Regulation by VK₃

G₁-Arrested Hep G2 were incubated with 0–50 μ M of VK₃ for 8 h, and whole cell extracts were prepared and subjected to immunoblotting for tyrosine-phosphorylated cdks (A; p-Cdk) and cyclins (B). Total cdk2 (panel A) and cdk1 (panel B) were used as loading controls. (C) Cyclin-cdk complexes were detected from the same cell lysate in panel B in an immunoprecipitating manner.

able to enhance G₂/M phase-dependent anticancer drugs for hepatoma chemotherapy. To demonstrate this hypothesis, combined treatment of VK₃ and anticancer drugs was administered for Hep G2 hepatocytes. In the presence of 5 μ M VK₃, the cytotoxicity of etoposide, a G₂ phase-dependent agent, was synergistically enhanced, especially at higher concentrations (Fig. 4, right panel). In contrast, VK₃ markedly reduced the cytotoxic activity of irinotecan, a S phase-dependent drug (left panel), at lower concentrations. These results indicated that VK₃ has potential as an enhancer of G₂ phase-dependent anticancer agents.

DISCUSSION

Our results indicate that VK₃ causes the dysfunction of Hep G2 cells by arresting the cell cycle in the G₂/M phase. Regarding the potential for VK₃ monotherapy, the cytotoxicity (IC₅₀ of 13.7 μ M) is not sufficient and additionally, it is hard to control the therapeutic range because of the steep gradient of the dose-dependent curve (Fig. 2A).

Since DNA is super-coiled, and tightly fitted in the chromosomes, the DNA-replication fork is unable to synthesize new DNA out of topological constrained DNA. Topoisomerase I relaxes the DNA molecules by binding to super-coiled DNA and cleaving a phosphodiester bond, resulting in a single-strand break.¹⁹ Inhibitors of topoisomerase I, including irinotecan, breaks may lead to cell cycle arrest in the S/G₂ phase and thus they are much more sensitive to S-phase synchronized cells.^{20,21}

On the other hand, topoisomerase II causes reversible double-strand DNA cleavage with the relaxation of DNA.²² It regulates over- and under-winding of the double helix and resolves nucleic acid knots and tangles. Topoisomerase II inhibitors, including etoposide, increase the steady-state con-

Fig. 4. Effect of VK₃ in the Cytotoxicity of Cell-Cycle Dependent Anti-cancer Agents

Cells were treated with irinotecan (left) or etoposide (right) for 24 h in combination with (●) or without (○) 5 μ M VK₃. Data were expressed as the mean $\pm$ S.D. ($n=5$). Synergicity was evaluated with two-way ANOVA, ** $p<0.01$.

centration of their covalent DNA cleavage complexes.^{22,23} For these reasons, etoposide induces cell cycle arrest in the G₂/M phase and apoptosis in a p53-related manner.²⁴

Our observations revealed that VK₃ accelerated S-phase turnover by the activation of cyclin A/cdk2 and induced G₂/M arrest by the inhibition of cyclin B/cdk1 complex formation (Fig. 3C). As a result, G₂/M-phase synchronized cells were accumulated and the cells were more sensitive to G₂/M-selective topoisomerase II inhibitor etoposide, as described above (Fig. 4). In contrast, the cells were less sensitive to irinotecan by the acceleration of S-phase turnover.

In conclusion, these results suggest the possibility of VK₃ as a chemotherapeutic enhancer in combination with topoisomerase II inhibitors. To evaluate the effects of the VK₃ combination with chemotherapeutic agents in detail, dose-response curves is not always suitable.²⁵ Further isobologram analyses are needed to clarify the VK₃ role is whether “additive” or “synergic”.^{26,27}

Acknowledgement This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

- 1) Cranenburg E. C., Schurgers L. J., Vermeer C., *Thromb. Haemost.*, **98**, 120–125 (2007).
- 2) Falanga A., *Pathophysiol. Haemost. Thromb.*, **33**, 389–392 (2003).
- 3) Lamson D. W., Plaza S. M., *Altern. Med. Rev.*, **8**, 303–318 (2003).
- 4) Falanga A., Marchetti M., Vignoli A., Balducci D., *Clin. Adv. Hematol. Oncol.*, **1**, 673–678 (2003).
- 5) Piccioli A., Falanga A., Prandoni P., *Semin. Thromb. Hemost.*, **32**, 810–813 (2006).
- 6) Juan C. C., Wu F. Y., *Biochem. Biophys. Res. Commun.*, **190**, 907–913 (1993).
- 7) Nishikawa Y., Carr B. I., Wang M., Kar S., Finn F., Dowd P., Zheng Z. B., Kerns J., Naganathan S., *J. Biol. Chem.*, **270**, 28304–28310 (1995).
- 8) Gant T. W., Rao D. N., Mason R. P., Cohen G. M., *Chem. Biol. Interact.*, **65**, 157–173 (1988).
- 9) Sun J. S., Tsuang Y. H., Huang W. C., Chen L. T., Hang Y. S., Lu F. J., *Cell Mol. Life Sci.*, **53**, 967–976 (1997).
- 10) Sata N., Klonowski-Stumpe H., Han B., Haussinger D., Niederau C., *Free Radic. Biol. Med.*, **23**, 844–850 (1997).
- 11) Markovits J., Wang Z., Carr B. I., Sun T. P., Mintz P., Le Bret M., Wu C. W., Wu F. Y., *Life Sci.*, **72**, 2769–2784 (2003).
- 12) Tamura K., Southwick E. C., Kerns J., Rosi K., Carr B. I., Wilcox C., Lazo J. S., *Cancer Res.*, **60**, 1317–1325 (2000).

- 13) Wang Z., Southwick E. C., Wang M., Kar S., Rosi K. S., Wilcox C. S., Lazo J. S., Carr B. I., *Cancer Res.*, **61**, 7211—7216 (2001).
- 14) Carr B. I., Wang Z., Wang M., Kar S., Wilcox C. S., Rosi K., Southwick E., Lazo J. S., *J. Mol. Biol.*, **326**, 721—735 (2003).
- 15) Sakamoto S., Yokoyama M., Zhang X., Prakash K., Nagao K., Hatanaka T., Getzenberg R. H., Kakehi Y., *Endocrinology*, **145**, 2929—2940 (2004).
- 16) Desai D., Wessling H. C., Fisher R. P., Morgan D. O., *Mol. Cell. Biol.*, **15**, 345—350 (1995).
- 17) Morgan D. O., *Nature* (London), **374**, 131—134 (1995).
- 18) Smits V. A., Medema R. H., *Biochim. Biophys. Acta*, **1519**, 1—12 (2001).
- 19) Hsiang Y. H., Liu L. F., *Cancer Res.*, **48**, 1722—1726 (1988).
- 20) Mathijssen R. H., Loos W. J., Verweij J., Sparreboom A., *Curr. Cancer Drug Targets*, **2**, 103—123 (2002).
- 21) Ray S., Shyam S., Fraizer G. C., Almasan A., *Mol. Cancer Ther.*, **6**, 1368—1378 (2007).
- 22) Hande K. R., *Eur. J. Cancer*, **34**, 1514—1521 (1998).
- 23) Hande K. R., *Biochim. Biophys. Acta*, **1400**, 173—184 (1998).
- 24) Nam C., Yamauchi H., Nakayama H., Doi K., *Neurotoxicol. Teratol.*, **28**, 664—672 (2006).
- 25) Berenbaum M. C., *Clin. Exp. Immunol.*, **28**, 1—18 (1977).
- 26) Steel G. G., *Int. J. Radiat. Oncol. Biol. Phys.*, **5**, 1145—1150 (1979).
- 27) Kano Y., Akutsu M., Tsunoda S., Mori K., Suzuki K., Adachi K. I., *Cancer Chemother. Pharmacol.*, **42**, 91—98 (1998).